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Absence of Relationship Between Vitamin A Alcohol Levels in Plasma and in Liver of Rats

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(*Received* 7 *August* 1952)

A moderately extensive amount of work has been reported in the literature dealing with the relationship between vitamin A levels in the blood and in the liver of various species. Generally speaking, these reports can be classified into two categories. Lewis, Bodansky, Falk & McGuire (1942) believe that plasma tends to resist, to a certain extent, any changes in its vitamin A concentration, despite wide variations in the amount stored in the liver, and this view has been supported by many investigators (Josephs, 1942; Horton, Murrill & Curtis, 1941; Brenner, Brookes & Roberts, 1942; Ralli, Baumann & Roberts, 1942).

On the other hand, Glover, Goodwin & Morton (1947), using a chromatographic separation of free and esterified vitamin A, have claimed that while the level of vitamin A in the blood cannot be deduced from the total concentration in the liver of rats, there is a rough linear relationship between the concentration of vitamin A alcohol in the liver and in the blood.

A qualitative difference in the distribution of vitamin A ester and alcohol and of carotenoids in the plasma of several species has recently been reported from this laboratory, and it has been suggested that specific proteins are involved in the absorption and transport of these compounds (Ganguly, Krinsky, Mehl & Deuel, 1952*b*). On account of the implications of this hypothesis, we have re-investigated the relationship between plasma and liver vitamin A alcohol levels in the rat. A preliminary report was presented to the American Society of Biological Chemists (Ganguly, Krinsky & Deuel, 1952*a*).

EXPERIMENTAL

Animals. Three lots of male rats of the University of Southern California strain were used in these experiments. The first lot (groups 1 and 2, Table 1) consisted of adult stock rats raised on Purina chow; the second lot (group 9, Table 1) was raised on a vitamin A-free diet (*U.S. Pharmacopoeia*, 1950) after weaning, and was used when depletion of vitamin A was indicated by cessation of growth. The third lot consisted of rats previously used for a vitamin A bioassay (*U.S. Pharmacopoeia*, 1950) of margarine samples; during the period of the experiment they had received 1·25–1·5 i.u. of vitamin A daily. These were used either immediately after the completion of the bioassay (animals for Fig. 1) or received weekly supplements of 10–15 i.u. of vitamin A until used (groups 3–8, Table 1). Although rats on a bioassay cannot be expected to grow normally, the small supplements given during the assay were enough to produce appreciable plasma vitamin A levels (see 0 hr. group in Fig. 1).

Supplements. The vitamin A solutions used for dosing were made from a fish-liver oil concentrate suitably diluted with peanut oil, mixed tocopherols, and soya lecithin to contain the requisite amounts of vitamin A, 5 mg. of tocopherols, and 40 mg. of lecithin/ml. of supplement. The solutions were administered by stomach tube.

Analysis. Blood was withdrawn by direct heart puncture, with an oxalated syringe, while the rats were under light Nembutal anaesthesia. The plasma, obtained by centrifugation, was extracted according to the method of Kimble (1939), and the other tissues were immediately excised, and were extracted following the procedure of Thompson, Ganguly & Kon (1949). The vitamin A alcohol and ester were separated by chromatography on alumina as described by Ganguly *et al.* (1952*b*), and were determined by the procedure of Thompson *et al.* (1949), except that a Coleman Junior spectrophotometer calibrated against a standard vitamin A solution was employed.

RESULTS

The rate of appearance of vitamin A alcohol and ester in blood and liver

Vitamin A-deficient rats, previously used for a vitamin A bioassay, were fasted for 12–18 hr. Each was given a single 1 ml. dose containing 14000 μg. of vitamin A ester. Groups were killed at given time intervals after dosing. The averages of the results of vitamin A analysis obtained from individual rats for liver and for plasma are presented in Fig. 1. The plasma ester showed the typical absorption curve, starting at 1 hr., rapidly reaching a peak at 5 or 6 hr., and then dropping sharply by the 15th hr. The liver ester presented a continual increase up to 18 hr., after which no further storage occurred.

The plasma alcohol increased at a slower rate than did the ester and, by the 24th hr., declined to the approximate pre-dosage level. On the other hand, the liver alcohol slowly increased, reaching a maximum at about 5 hr., and maintained a fairly steady

* Procter and Gamble Fellow.

Table 1. *The relation between vitamin A alcohol in the plasma and vitamin A alcohol and ester in the liver of male rats receiving various diets and supplements*

(The figures in brackets indicate the range of values obtained.)

Group	No. of rats	Weight range (g.)	Age	Dietary regime: Diet	Dietary regime: Treatment	Vitamin A alcohol: Plasma (μg./100 ml.)	Vitamin A alcohol: Liver (μg./g.)	Vitamin A ester. Liver (μg./g.)
1	24	218–435	Adult	Purina chow	None	26·9 (19·9–36·2)	21·7 (1·2–61·4)	473 (154–794)
2	26	245–407	Adult	Purina chow	110000 μg. vitamin A divided into 13 daily doses	26·2 (14·4–38·1)	57·4 (10·9–234·0)	3470 (1595–5520)
3	16*	149–338	3–5 months	Vitamin A-free	3–21 days on Purina chow	23·5 (17·3–28·8)	4·4 (1·5–8·0)	178 (17–307)
4	9*	100–222	3–5 months	Vitamin A-free	110000 μg. vitamin A divided into 4 daily doses	17·4 (13·6–20·2)	169·3 (137·9–186·0)	2033 (1920–2118)
5	20*	110–270	3–5 months	Vitamin A-free	3300 μg. vitamin A in one dose	20·1 (14·1–25·5)	16·3 (5·7–30·0)	332 (182–442)
6	20*	136–255	3–5 months	Vitamin A-free	8300 μg. vitamin A in one dose	24·4 (17·3–29·5)	28·2 (5·6–61·6)	638 (316–1275)
7	20*	127–245	3–5 months	Vitamin A-free	16600 μg. vitamin A in one dose	23·9 (19·1–29·0)	29·3 (8·2–81·0)	824 (477–1258)
8	44*	65–188	3–5 months	Vitamin A-free	22–48 μg. vitamin A in one dose	27·0 (19·4–36·7)	0·1 (0·0–0·4)	0·4 (0·0–1·4)
9	13*	121–203	2 months	Vitamin A-free	None	0	0	0

* Three to four rats pooled.

level throughout the 24 hr. period, in spite of the continual increase in the liver ester. It appears that under these conditions the liver vitamin A alcohol concentration rapidly attains a steady level, even while there is a continual deposition of vitamin A ester in the liver.

from 0·1 to 169·3 μg./g., the plasma vitamin A alcohol remained fairly stable, namely 17·4–27·0 μg./100 ml. It is noticeable that group 4, which had the highest liver vitamin A alcohol content, was accompanied by the lowest plasma level of this component, whereas group 8, in which the livers contained only

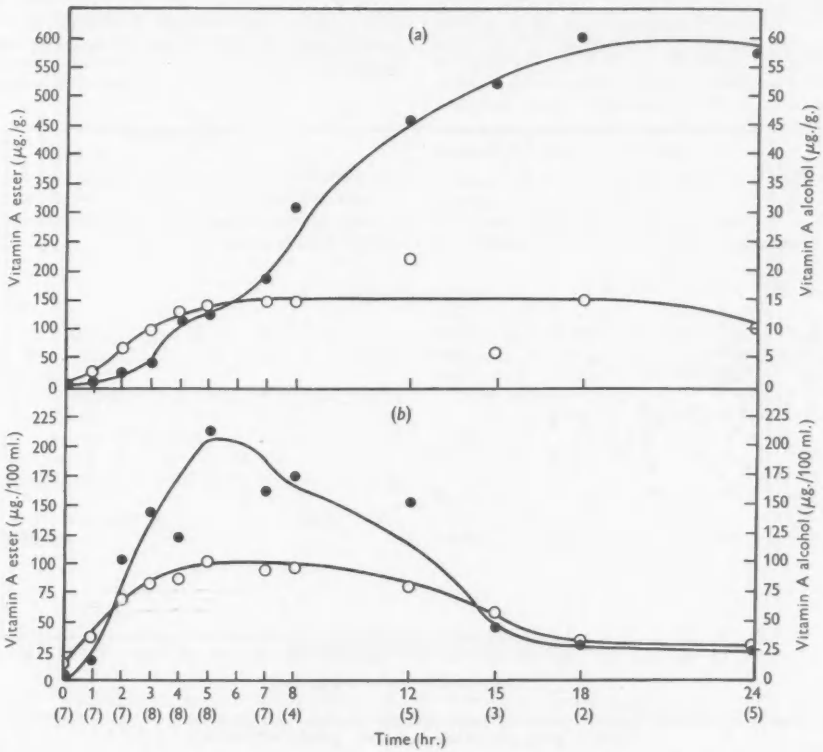
(a)
(b)
Vitamin A ester (μg./g.)
Vitamin A alcohol (μg./g.)
Vitamin A ester (μg./100 ml.)
Vitamin A alcohol (μg./100 ml.)
0 1 2 3 4 5 6 7 8 12 15 18 24
(7) (7) (7) (8) (8) (8) (7) (4) (5) (3) (2) (5)
Time (hr.)

Fig. 1. The rate of appearance of vitamin A ester and alcohol in (*a*) liver and (*b*) plasma of vitamin A-deficient male rats following a single 1 ml. dose containing 14000 μg. vitamin A, 5 mg. mixed tocopherols, and 40 mg. lecithin. The figures in brackets indicate the number of animals upon which the averages were determined. ●—●, Vitamin A ester; ○—○, vitamin A alcohol.

The relationship between plasma and liver vitamin A alcohol

In order to obtain wide variations in the concentration of vitamin A in the liver, several groups of rats were given different diets and supplements, as described in Table 1. All animals were killed not earlier than 3 days after their last supplement following a 12 to 18 hr. fast. The results are summarized in Table 1, in which the averages of the individual values within each group are presented. Despite the variation in the liver vitamin A alcohol traces of vitamin A alcohol, had the highest plasma concentration of this alcohol.

Fig. 2 represents the individual plasma alcohol values plotted against their respective liver alcohol values for the rats used in Table 1. This diagram does not show any direct relationship between liver and plasma vitamin A alcohol. These results, however, confirm the earlier observations of Lewis *et al.* (1942) and Glover *et al.* (1947), that normal plasma vitamin A concentration can be maintained even when there is none in the liver. This blood vitamin A disappears on prolonged depletion.

Vitamin A in kidneys

In normal adult rats, small amounts of vitamin A alcohol, 0·4–1·5 μg./g. tissue, were present in all kidneys, whereas the ester form was found only occasionally, at approximately the same concentration as the free form. Vitamin A alcohol, 5·2 μg./g. and vitamin A ester, 66·9 μg./g., were found in the kidneys of vitamin A-deficient rats 24 hr. after a single dose of 14 000 μg. of vitamin A had been given.

reactions. The average liver alcohol value of the females was 35·8 μg./g. tissue (range 3·1–86·4 μg./g. tissue) and the liver ester value was 714 μg./g. tissue (range 83·0–1168 μg./g. tissue). Although we cannot compare these females directly with the male rats of group 1, Table 1, as their previous dietary intake of vitamin A is unknown, we feel it necessary to emphasize the fact that these females with higher liver vitamin A alcohol stores had lower plasma alcohol values as compared with the males.

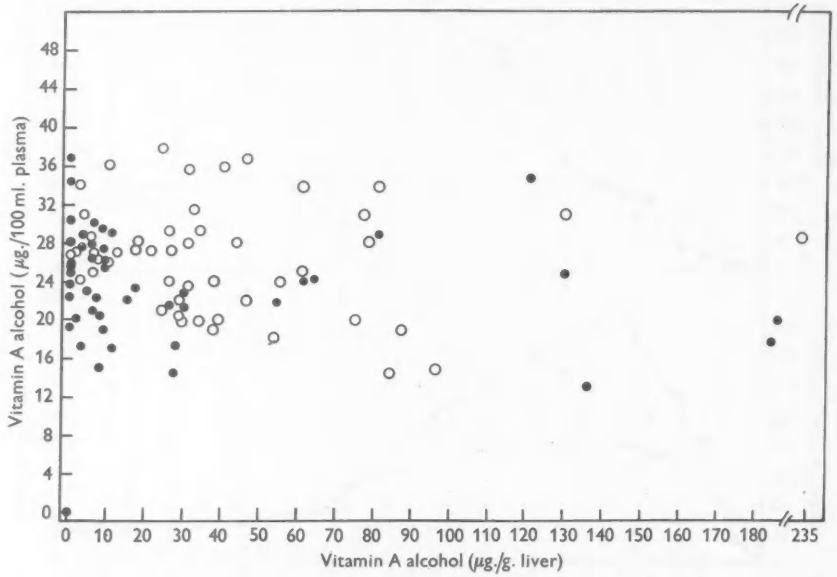

Fig. 2. Relationship between liver and plasma vitamin A alcohol in rats. ○ represents the animals in groups 1 and 2, Table 1; ● represents the animals in groups 3–9, Table 1.

Sex difference

Moore, Sharman & Ward (1951) have recently reported a sex difference in the total vitamin A contents of blood and of liver of rats. The males had more vitamin A in the blood and less in the liver, compared with the female rats. More recently, Booth (1952) has reported a similar sex difference in liver storage in rats. The vitamin A ester and alcohol contents of the plasma and liver of fourteen female stock rats were determined. Despite the fact that comparable amounts of plasma were analysed in both stock male and female rats, the values for the female rats were so low that a definite Carr-Price reaction could be obtained in only one case, whereas in the males all plasma samples gave positive

DISCUSSION

Our results do not show any direct relationship between the plasma and the liver vitamin A alcohol, as suggested by Glover *et al.* (1947). There are, however, two major deviations in the techniques employed. The first is the method of extraction. We have compared the two methods but found no significant difference. The second involves the instrumental determination of the rapidly fading blue colour developed by the Carr-Price reaction. In our procedure, a direct reading instrument, such as the Coleman spectrophotometer, should give a more precise value than could be obtained with the Beckman spectrophotometer, which necessitates time-consuming adjustments during the measurements.

On the other hand, these results demonstrate that plasma tends to maintain a rather steady vitamin A alcohol level. Although this mechanism is still unknown, one possible explanation is that the plasma concentration is dependent upon the degree of association of vitamin A alcohol with a protein carrier. Variations in this degree of association may explain the temporary increase in plasma vitamin A alcohol immediately following absorption of vitamin A. The sharp increase in the ester form of vitamin A during active absorption may likewise be due to its association with another protein.

SUMMARY

1. The rate of appearance of vitamin A ester and alcohol in plasma and liver, respectively, of vitamin A-depleted male rats following a single dose of vitamin A was studied. In plasma, the ester form presented a typical sharp peak, and the free form showed relatively less increase. In liver, the ester form continued to be deposited up to 18 hr. after dosing, whereas the alcohol form increased for only 5 hr. and was then maintained at a fairly constant level.

2. In separate experiments, normal stock and vitamin A-depleted male rats were used either as such or after vitamin A dosage, to determine the vitamin A content in plasma and in liver. A direct relationship between the plasma and the liver vitamin A alcohol could not be confirmed, inasmuch as, despite wide variations in the concentrations of vitamin A alcohol in the liver, the plasma level remained relatively constant.

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Acetylation of Collagen

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In recent years it has been possible to account for almost the whole nitrogen content of collagen in terms of its constituent amino-acids (Bowes & Kenten, 1948*a*). From the known composition of collagen it is evident that many of its side chains should be distinguished by polar groups, of which the most important are carboxyl, amino, guanidino and aliphatic hydroxyl groups. Before asking which of these groups may be responsible for such complicated changes as occur in reaction with tanning agents, it seems desirable to inquire whether they are free to react quantitatively with simple chemical reagents. It has already been shown by Bowes & Kenten (1949) that the carboxyl groups can be quantitatively esterified, and one of us (Green, 1951) has demonstrated a stoicheiometric relationship when dry hydrogen chloride reacts with the protein. We report here a detailed study of the reaction between collagen and acetic anhydride.

This substance might be expected to react with some or all of the amino, guanidino and hydroxyl groups. Olcott & Fraenkel-Conrat (1947) found that several proteins in aqueous alkaline solution reacted with acetic anhydride in an amount equivalent to their free amino groups. They recommended this reaction as a specific means for the *N*-acetylation of proteins. Several workers have found that, when the conditions are changed and the solid protein is treated with acetic anhydride, both amino and hydroxyl groups are substituted. This was the case

when solid insulin reacted with acetic anhydride, either in the presence or absence of pyridine (Freudenberg & Dirscherl, 1928; Jensen & De Lawder, 1930). Hendrix & Paquin (1938) obtained similar results with casein, egg albumin and edestan. Other investigators have used boiling acetic anhydride under conditions which might be expected to induce undesirable side reactions. It will be shown here that, by modifying the experimental conditions, it is possible to introduce into collagen a number of *N*-acetyl groups exactly equivalent to the reported free amino groups, or to effect a higher degree of acetylation in which about 80% of the hydroxyamino-acid residues are also involved.

MATERIALS

The collagen used was standard hide powder. Denatured collagen was prepared by immersing hide powder in water at 72° for 5 min., filtering, washing with cold water, dehydrating with acetone, drying in the air of the laboratory and grinding to pass a 40 mesh/in. sieve. Acetic acid and acetic anhydride were of A.R. grade. Anhydrous acetic acid was made by refluxing glacial acetic acid with the calculated quantity of acetic anhydride and then distilling with the usual precautions to exclude moisture. The distillate was used in the preparation of water-free mixtures of acetic acid and acetic anhydride, which were then analysed by the iodometric method of Orton & Bradfield (1927). The acetic anhydride itself was found to contain about 6% acetic acid.

METHODS

Acetylation in aqueous suspension

A method similar to that of Olcott & Fraenkel-Conrat (1947) was used. Collagen was soaked for 24 hr. in half-saturated sodium acetate solution below 10°. It was then stirred vigorously for 1 hr. while 10% by weight of acetic anhydride was added in small portions. The pH was held near 8·0 by adding NaOH solution, and the temperature was kept below 10°. The product was then filtered and freed from sodium acetate by washing with frequent changes of distilled water for 1 week. Thorough washing was essential, as the method described below for the determination of total combined acetyl groups would give a high result if any sodium acetate were still held by the collagen fibres. That washing for 1 week was sufficient was proved by analysing the product, washing for 1 week more and analysing again. The results of the two analyses were found to be identical and were therefore taken to represent combined acetyl. The washed product was dehydrated with several changes of acetone and then exposed to the air of the laboratory. It was found that prolonging the reaction beyond 1 hr. did not increase the degree of acetylation. However, re-acetylation of the washed and dried product did introduce more acetyl groups. After two acetylations there was no further increase. A few experiments in other aqueous media are reported in Table 1 (see Results).

Acetylation of collagen in non-aqueous media

It had already been observed (Green, 1951) that completely dry collagen could not be acetylated with cold acetic anhydride. Hendrix & Paquin (1938), however, report having acetylated several proteins containing not less than 7% water. This indicated that the water content of the collagen might be important. We therefore used the isopiestic method of Robinson & Sinclair (1934), as previously described (Green, 1948), to prepare a range of samples of collagen extending from the perfectly dry material to a water content of 100% of the dry weight.

(*a*) *Acetylating medium free from water.* The samples were covered with 6 parts of acetic anhydride, calculated on the dry protein weight, and kept at laboratory temperature (30°) for periods ranging from a few hours to 9 days. They were then filtered, washed with several changes of acetone and extracted with acetone in a Soxhlet apparatus for 48 hr. Preliminary experiments had shown that, after the first day's extractions, no further change in the analytical figures occurred even after several days in the extraction apparatus. Prolonged washing with cold acetone led to the same result.

(*b*) *Acetylating medium and collagen both free from water.* Since the action of water in promoting acetylation might be through the medium of acetic acid, a series of experiments was carried out in the absence of water. Collagen was dried at 100° *in vacuo* over CaO and treated with water-free mixtures of acetic anhydride and acetic acid. After acetylation the products were washed and extracted with acetone as before. As a test of the reliability of the method of preparation for analysis, samples of dry collagen were soaked for 3 days in either pure acetic acid or pure acetic anhydride. Each, after extraction and analysis by the methods described below, gave a negligible value for combined acetyl groups. Any possibility that the acetyl figures reported in the next section might be due, even in part, to adsorbed acetic acid is thus excluded.

In another series, to demonstrate the relationship between the two principal methods, collagen was acetylated in alkaline suspension, dried and subjected to the action of a 1:1 mixture of acetic anhydride and acetic acid.

Finally, a series of experiments was made in which collagen of varying water content was acetylated with the same 1:1 mixture.

In all these experiments the weight of the acetylating mixture was six times the protein dry weight.

Analysis of products

Total acetyl. Acetylated collagen (0·5 g.) after drying in the air oven at 103°, was heated under reflux for 2 hr. with 50 ml. 2N-H_2SO_4. The mixture was then distilled in a current of steam until its volume had been reduced to about one-half, and about 500 ml. had been collected in the receiver. The distillate was titrated with 0·05N-NaOH, and from the result was subtracted a small blank obtained by a similar hydrolysis and distillation with unacetylated collagen.

N-Acetyl. Acetyl groups in proteins may be attached to either oxygen or nitrogen, and it is generally believed that the two forms of combination can be differentiated by their stability to alkali (Hendrix & Paquin, 1938). All those acetyl groups which can be hydrolysed by cold dilute NaOH are believed to be attached to oxygen, whilst *N*-acetyl groups can be removed only by boiling in dilute acid solution.

This principle, which was adopted by Blackburn & Phillips (1944), is supported by the observations of Synge (1939) on amino-acids and of Wolfrom, Konigsberg & Soltzberg (1936) on sugars; but the means of its experimental

application to proteins must be chosen with care. Blackburn & Phillips, in their work on wool, immersed acetylated derivatives in excess alkali and then back-titrated in the absence of protein to pH 7–8. The alkali used up, corrected by the result of a similar titration with the original protein, was regarded as an approximate measure of the extent of *O*-acetylation. However, this use of a blank may be subject to some uncertainty, since *N*-acetylation will displace the pH titration curve of the protein, making it difficult to define the state of the ionizable groups before the addition of alkali. A sharp distinction between *N*- and *O*-acetyl groups is not easily achieved.

A more reliable application of the principle consists in saponification of the *O*-acetyl groups with excess dilute alkali, followed by removal of the soluble acetate by dialysis or, for insoluble proteins, by simple washing. The acid hydrolysis and distillation procedure applied to the residue then gives an accurate figure for the degree of *N*-acetylation. This method suffers from the very long period required for washing or dialysis of swollen or gelatinous proteins, and is unsuitable for the examination of large numbers of samples. It was used here only for confirmation of the method finally adopted, in which the *N*- and *O*-acetyl groups were differentiated by measurements of free amino N.

Free amino N. This was determined by a modification (Doherty & Ogg, 1943) of the manometric Van Slyke procedure, using samples which passed a 40 mesh/in. sieve.

Total N. This was determined by a Kjeldahl method.

RESULTS

Acetylation in aqueous suspension

Collagen was treated with acetic anhydride after it had been soaked in solutions of various degrees of acidity or alkalinity. In only one case was any *O*-acetylation detected. Table 1 shows that the most successful method of *N*-acetylation entailed the use of sodium acetate solution at pH 8, as recommended by Olcott & Fraenkel-Conrat (1947) for soluble proteins. To attain the highest degree of *N*-acetylation, it was always necessary to repeat the treatment. Prolonging the time or increasing the amount of acetic anhydride added in a single acetylation was not enough. Table 2 shows that the maximum degree of *N*-acetylation attainable with heat-denatured collagen is the same as that for the original protein, namely 0·40 m-mole/g. protein.

Table 1. *Action of acetic anhydride on collagen in aqueous suspension*

(Composition of derivatives expressed as m-moles/g. original dry collagen.)

Nature of suspension	*O*-Acetyl	*N*-Acetyl	Amino N (Van Slyke)*
N-Acetic acid	Nil	0·05	0·31
Water	0·15	0·05	0·31
Sodium acetate (pH 8)			
First acetylation	Nil	0·24	0·12
Second acetylation	Nil	0·36	0·01
Third acetylation	Nil	0·38	0·00
Fourth acetylation	Nil	0·38	0·00
0·02 N-Sodium hydroxide	Nil	0·18	0·17

* Amino N for dry collagen = 0·35 m-mole/g.

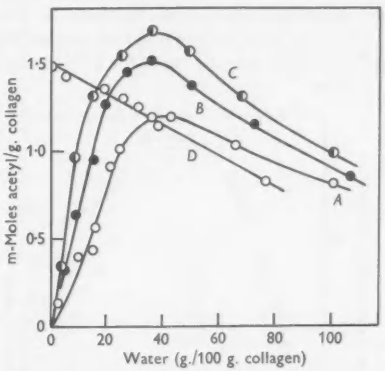

Fig. 1. Effect of initial water content of collagen on degree of total acetylation by: *A*, acetic anhydride for 1 day; *B*, acetic anhydride for 3 days; *C*, acetic anhydride for 5 days; *D*, mixture of equal parts acetic anhydride and acetic acid for 3 days.

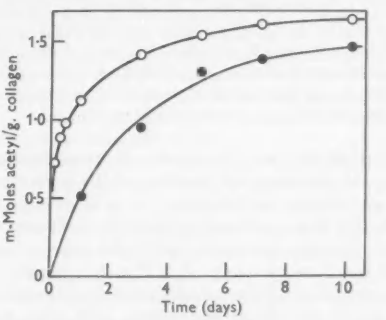

Fig. 2. Acetylation of collagen: ●, action of acetic anhydride on collagen containing 16% water; ○, acetic anhydride on collagen containing 36% water.

Acetylation in non-aqueous media

Method (*a*). The action of excess acetic anhydride on collagen containing relatively small quantities of sorbed water introduced a much greater number of acetyl groups, many of which could be removed by dilute sodium hydroxide; these, however, were split off only very slowly by treatment with milder alkalis at pH 8–9. The more extensively acetylated specimens swelled noticeably during the reaction and after drying resembled heat-denatured collagen in appearance. Apart from this, the acetylated products retained their fibrous nature and their colour was unchanged.

Fig. 1 (curves *A*, *B*, *C*) shows the effect of initial water content of the collagen on the total degree of acetylation for various reaction times. In Fig. 2 are curves showing the rate of total acetylation of collagen of two different water contents. At 36% water, corresponding to the maxima of Fig. 1, acetylation appears to reach saturation at 1·7 m-moles/g. collagen, but the curve for 16% water rises only slowly.

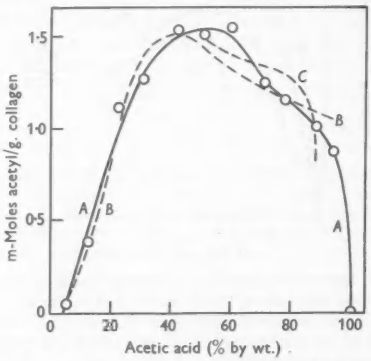

Fig. 3. Degree of total acetylation of collagen by mixtures of acetic anhydride and acetic acid: *A*, oven-dry collagen for 3 days; *B*, calculated from curve *B* of Fig. 1 on the assumption that the water contained in the collagen reacts rapidly with the acetic anhydride and has no other influence on the course of reaction; *C*, calculated from curve *D* of Fig. 1 on the same assumption.

Method (*b*). Two other series of experiments were made to elucidate the function of the sorbed water in promoting acetylation. It was found that, although pure acetic anhydride did not react with dry collagen, acetylation did take place if acetic acid was present; curve *A* of Fig. 3 shows the total degree of acetylation achieved in 3 days by mixtures of acetic anhydride and acetic acid. The broad, flat maximum near 50% acetic acid indicates that the most effective acetylating agent for dry collagen is a mixture of approx. equal parts of the anhydride and the acid. When the acetylating mixture already has this optimum composition, curve *D* of Fig. 1 shows that any sorbed water in the collagen retards the reaction.

A more detailed picture of the reaction is presented in Figs. 4 and 5, setting out the rates of *N*- and *O*-acetylation for different reaction mixtures. A few experiments with denatured collagen in the 1:1 acetic anhydride-acetic acid mixture gave results, identical with those for native collagen, which have been omitted from the figures for the sake of clarity. For the same reason we have omitted from Fig. 5 the experimental points derived from the action of the same acetylating mixture on collagen previously acetylated at pH 8. The composition of the end products of these two series appears in Table 2.

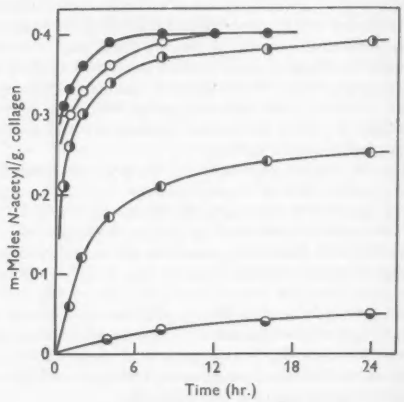

Fig. 4. Degree of *N*-acetylation of dry collagen by mixtures of acetic anhydride and acetic acid (w/w): ◒, 10% acetic acid; ◐, 30% acetic acid; ◑, 50% acetic acid; ●, 70% acetic acid and 85% acetic acid (experimental points for these two mixtures were indistinguishable); ○, 95% acetic acid.

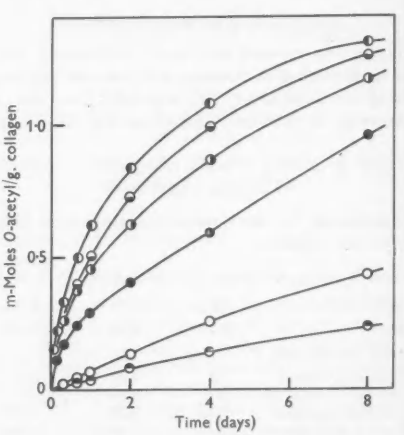

Fig. 5. Degree of *O*-acetylation of dry collagen by mixtures of acetic anhydride and acetic acid (w/w): ◓, 10% acetic acid; ◒, 30% acetic acid; ◐, 50% acetic acid; ◑, 70% acetic acid; ●, 85% acetic acid; ○, 95% acetic acid.

In Figs. 4 and 5, the degree of *N*-acetylation was obtained from a comparison of the Van Slyke amino-nitrogen values of the acetylated product and the original protein. Several determinations on collagen and on denatured collagen gave the value of 0·35 m-mole free NH_2/g. protein, in good agree-

Table 2. *Composition of collagen derivatives containing the maximum proportion of acetyl groups related to the amino-acid composition of collagen (after Bowes & Kenten, 1948a)*

Starting material	Method of acetylation	Total N (%)	*N*-Acetyl (m-moles/g. collagen)	*O*-Acetyl (m-moles/g. collagen)
Collagen	None	18·0	0	0
	Alkaline suspension (I)	17·6	0·40	0
	Mixture of acetic acid and acetic anhydride (II)	16·6	0·40	1·33
	(I) followed by (II)	16·5	0·40	1·36
Denatured collagen	None	17·8	0	0
	I	17·3	0·40	0
	II	16·6	0·40	1·36

Composition of collagen

Amino-acid	m-Moles/g. collagen	Amino-acid	m-Moles/g. collagen
Lysine	0·31	Serine	0·33
Hydroxylysine	0·08	Threonine	0·20
Arginine	0·51	Hydroxyproline	1·07
Histidine	0·05	Tyrosine	0·08

ment with that reported by Bowes & Kenten (1948*a*). However, Table 1 shows that acetylation of collagen at pH 8 introduces 0·40 m-mole acetyl/g., and none of these groups is split off by dilute sodium hydroxide. They may therefore be regarded as being attached to amino nitrogen, suggesting as a more accurate value for the free amino groups of collagen, 0·40 m-mole/g. This is supported by the known proportions of lysine and hydroxylysine in the protein. The use of the empirical extrapolation procedure of Doherty & Ogg (1943) thus apparently leads to a figure for collagen which is too low by about 12%. The results for *N*-acetylation reported here have been derived by multiplying the observed Van Slyke figures by a factor of 1·14 and subtracting from 0·40. The *O*-acetyl values are then taken as the difference between total and *N*-acetyl. This method of calculation leads to good agreement with the more time-consuming method of alkaline hydrolysis described above.

It will be observed that the total degree of acetylation at saturation is the same, whether the starting material is collagen or *N*-acetylcollagen, and whether the acetylating agent is acetic anhydride in the presence of water or mixed with anhydrous acetic acid. Table 2 presents the composition of collagen derivatives containing the maximum proportions of *N*- and *O*-acetyl groups.

DISCUSSION

The collagen preparation used here had been made from limed hide and was therefore not the same as the material, prepared with a minimum of chemical treatment, whose composition has been summarized by Bowes & Kenten (1948*a*). However, our collagen corresponded almost exactly in both total nitrogen and amino nitrogen to an alkali-treated collagen which the above authors showed, in a second paper (Bowes & Kenten, 1948*b*), to differ materially from the original only in the number of amide groups. We shall assume, therefore, that the basic and hydroxyamino-acid content of our collagen is the same as that reported by Bowes & Kenten, part of whose data we reproduce here. In Table 2 are shown the amounts of those amino-acids whose side chains might be expected to react with acetic anhydride.

Although free amino-acids are easily acetylated at the α-amino group, the distal groups of the basic amino-acids are more resistant to attack. Neuberger (1938) found that arginine and histidine in mixtures of acetic anhydride and pyridine reacted at the α-amino group only. Bergmann & Köster (1926) found that acetic anhydride with arginine gave the α-acetyl derivative in the cold and a 'triacetyl anhydroarginine' only at 100°. There appears to be no record of simple acetylation of arginine at the guanidino group or of histidine at the glyoxaline group. However, Synge (1939) prepared αε-diacetyllysine by the action of acetic anhydride in alkaline solution, so that, of the basic amino-acids, it would seem that only lysine and hydroxylysine are likely to be easily acetylated in the side chain under mild conditions; this may apply to their residues in the protein, as well as to the free amino-acids.

We have already pointed out that acetylation of collagen in alkaline suspension quickly introduces alkali-stable acetyl groups to the extent of 0·40 m-mole/g. protein, unchanged by repetition of this treatment. Simultaneously, the amino-nitrogen figure falls to zero. Since the number of groups intro-

duced is exactly equivalent to the sum of lysine and hydroxylysine residues shown in Table 2, it is clear that acetic anhydride in slightly alkaline suspension must react quantitatively with the free amino groups of collagen and with no others.

The reaction of dry collagen with acetic anhydride-acetic acid mixtures is more complex. Figs. 4 and 5 may be summarized by saying that the rate of *N*-acetylation is highest and almost constant in mixtures containing from 5 to 50 % acetic anhydride, and under these conditions *N*-acetylation is complete in a few hours; *O*-acetylation becomes appreciable only when the proportions of acid and anhydride are of the same order, and requires more than 4 days for completion. The combination of these two effects accounts for the shape of curve *A* in Fig. 3. The close agreement of the results for collagen and for previously *N*-acetylated collagen shows that the same nitrogen atoms are taking part in the reaction with acetic anhydride whether the medium is aqueous alkali or anhydrous acetic acid.

It may be pointed out that, in a 5 % solution of acetic anhydride in acetic acid, *N*-acetylation is complete in 8 hr., while *O*-acetylation has hardly begun (0·02 m-mole/g.). This provides an alternative to the method of Olcott & Fraenkel-Conrat (1947) for preparing *N*-acetylated proteins, and has the advantage that the degree of acetylation is more easily controlled.

The maximum number of alkali-labile acetyl groups introduced is 1·33 m-moles/g., a figure which has been confirmed in several independent experiments. It is unaffected by previous heat denaturation of the protein. It is most improbable that these groups have been introduced by reaction between acetic anhydride and the free carboxyl groups of the aspartic and glutamic acid residues. Not only is the number of acetyl groups far too great to be accounted for in this way, but any mixed anhydrides resulting from a hypothetical reaction of this kind would be expected to resemble the simpler acid anhydrides and to be easily hydrolysed by water and mild alkalis. On the other hand, the easy removal of the acetyl groups by 0·05 N-sodium hydroxide makes it almost certain that they are present as esters of alcoholic hydroxyl groups.

Sakami & Toennies (1942) prepared the *O*-acetyl derivatives of serine, threonine, tyrosine, and hydroxyproline, and report that all were rapidly hydrolysed by dilute alkali. Synge (1939) prepared *ON*-diacetyltyrosine and found that the *O*-acetyl group was completely split off by excess 0·005 N-sodium hydroxide, while the *N*-acetyl group was unaffected. These considerations, taken with the fact that the only other possibility, arginine, is difficult to acetylate at the guanidino group and, in any case, is not present in sufficient quantity, lead us to the conclusion that the alkali-labile acetyl groups introduced into collagen are attached to the oxygen of the hydroxyamino-acids. Their number, 1·33 m-moles/g., is less than the total number, 1·73 m-moles/g. of these residues, indicating that about one in four of the hydroxyl groups of collagen is not free to react. This ratio is maintained even after denaturation, and emphasizes the fact that, whatever changes may occur during denaturation, they do not involve the availability of amino or hydroxyl groups.

The shape of the curves in Fig. 1 shows that sorbed water must play a double role in acetylation by acetic anhydride. Oven-dry collagen does not react with the anhydride, but small quantities of water have a catalytic effect, which is masked by dilution or hydrolysis of the anhydride when larger quantities of water are present. Since in the absence of water the curve for acetic anhydride-acetic acid mixtures in Fig. 3 also shows a maximum, it is relevant to inquire whether the water has any direct catalytic action or whether the only catalyst is acetic acid.

If the acetylating agent is a mixture of equal parts of acetic acid and acetic anhydride, corresponding to the maximum of Fig. 3, the presence of any quantity of water in the collagen diminishes the rate of reaction. This suggests that the only function of the water is to form acetic acid, which acts as a catalyst when it is present in small amount, but as a diluent when present in large amount, as in the experiments of Fig. 1*D*.

This presumption can be tested further by comparing the data of Fig. 1, curve *B*, with Fig. 3, curve *A*. The reaction of acetic anhydride with liquid water is almost complete in a few hours, and it is conceivable that, with water adsorbed on a regular surface which also has an affinity for the anhydride, hydrolysis may be much more rapid. We shall assume, then, that the collagen-bound water in the experiments of Fig. 1, *B* and *D*, reacts with acetic anhydride much more rapidly than does the protein. Any point from these experiments with collagen + acetic anhydride + water can then be recalculated as a mixture of collagen + acetic anhydride + acetic acid, and as such plotted in Fig. 3. The two broken curves are derived in this way. It is evident that they follow the principal curve of Fig. 3 over almost the whole range. The comparison fails only when the collagen contains quantities of water almost equivalent to the total anhydride used. Then the competition between protein and water for the anhydride becomes apparent and the curves diverge. The close agreement between the two methods of acetylation when reduced to a common basis, even by this approximate method, makes it clear that the catalyst is acetic acid and that the water is only an intermediate.

SUMMARY

1. In mildly alkaline suspension collagen is acetylated by acetic anhydride at the free amino groups. The number of *N*-acetyl groups introduced is equal to the total number of lysine and hydroxylysine residues found by analysis.

2. Dry collagen does not react with cold acetic anhydride, but in the presence of acetic acid or of small amounts of water both *N*- and *O*-acetylation take place.

3. The reaction is a slow one and is catalysed by acetic acid or, indirectly, by water. At its completion all the ϵ-amino groups and about 77% of the hydroxyamino-acid residues of the protein have reacted.

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The Role of the Amino and Hydroxyl Groups of Collagen in Chrome Tanning

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The reaction between collagen and chromic salts, whereby small quantities of the latter bring about profound changes in the physical and chemical properties of the former, is of great practical importance in the manufacture of leather and of theoretical interest as an example of the combination of proteins with metals. It has been the subject of many researches, which have been discussed in a recent review by Gustavson (1949). Both reactants are highly complex systems and, while many investigators have studied the mechanism of the reaction by modifying the chromic solution, the complementary method of attack, modification of the protein, has usually been restricted to changes which arise directly from the processes of manufacture. Attempts to measure the effect of simple chemical changes in the collagen, controlled in extent and limited to a specific group in the protein molecule, have been less numerous.

An outstanding example of the latter approach is the work of Bowes & Kenten (1949), who esterified the free carboxyl radicals of collagen and showed that these groups are of primary importance in the reaction with chromium. However, simple salt formation between chromium and carboxyl is not sufficient to explain the marked increase in stability accompanying chrome tanning, particularly when we remember the ease of hydrolysis of chromic salts. It is now firmly established that the chromic salt must form, by means of its polynuclear co-ordination complexes, a bridge or network of covalent and co-ordinate links between reactive points on the same or neighbouring protein chains.

The data presented by Bowes & Kenten (1949) show that carboxyl and amino groups constitute many of these reactive points. This is in conformity with the common belief that the reactions of chrome tanning are almost exclusively associated with acidic and basic protein groups. No evidence has been published to suggest that the numerous hydroxyl groups of collagen might also be involved. In this paper we describe the use of acetylated

collagen to confirm the findings of Bowes & Kenten for the amino group and to show that aliphatic hydroxyl groups also play a major role in the fixation of chromium.

METHODS

Collagen and denatured collagen were the same as those used in the previous paper (Green, Ang & Lam, 1953).

Acetylation of collagen. This was carried out by the methods also described in the previous paper. *N*-Acetylcollagen resulted from the action of acetic anhydride on an alkaline suspension of the protein; and a complete range of *N*-acetylated samples was made by the action of a 5% (w/w) solution of acetic anhydride in acetic acid on dry collagen for periods varying from 30 min. to 8 hr. None of these preparations contained any measurable quantity of *O*-acetyl groups.

A further series of derivatives was made by treating fully *N*-acetylated collagen with a mixture of equal parts acetic anhydride and acetic acid in the absence of water, resulting in the acetylation of up to 80% of the hydroxyamino-acid residues.

One of these mixed acetyl derivatives was immersed overnight in excess 0·1 N-NaOH below 10° to remove *O*-acetyl groups. It was then brought to equilibrium at pH 4 by means of acetic acid and washed for 1 week with distilled water. The product was still fully *N*-acetylated but contained no *O*-acetyl groups.

Cationic chromium solutions. Normal chromic sulphate was dissolved in cold water and made basic by slow addition, over a period of 2 days, of the calculated quantity of aqueous NaOH. The solutions were then diluted to 0·167 M, i.e. to 25·3 g. Cr_2O_3/l. and allowed to age for 2 months. In this way basic chromic sulphate solutions were made, ranging from the normal salt to one in which one-half of the sulphate radicals had been replaced by basic groups. The basic solutions, of course, contained Na_2SO_4 in amount increasing with the basicity. We believe that the use of aged basic chromic solutions is to be preferred to the practice of adding alkali to the experimental solution while tanning is in progress, since this may result in precipitation, particularly when the pH is raised above 3·5. The solutions used here, with pH values from 1·8 to 3·3, covered the whole range of basicities employed in commercial tanning.

Anionic chromium solution. A solution of sodium oxalatochromiate was prepared by reducing hot sodium dichromate solution with the theoretical quantity of oxalic acid, and the pH of the product was adjusted to 5·0 by the gradual addition of NaOH. It was diluted to a concentration of 25·3 g. Cr_2O_3/l., allowed to age for 3 months and filtered before use.

Chrome tanning. Collagen or acetylated collagen was dried in the air oven at 103°, and 1 g. was then soaked in 10 ml. distilled water for 24 hr. at room temperature (30°). Chromium solution (25 ml.) was added and the mixture was allowed to stand, with frequent agitation, for periods varying from 4 hr. to 4 days. The pH of the cationic chromic solution rose by 0·1–0·2 during the experiment. At the end of the reaction period the chromed collagen was filtered, washed for 2 days to free it from uncombined chromium, dried in the oven at 103° and analysed.

Analysis. Collagen and its derivatives before reaction with chromium were analysed for *N*- and *O*-acetyl as described in the previous paper. No attempt was made to measure the Van Slyke amino N after reaction with chromium, but the total acetyl figure was determined as before.

Chromium was estimated by drying at 103°, ashing, heating the ash with a mixture of Na_2CO_3 and MgO, and estimating the chromate by titration against ferrous ammonium sulphate in the presence of *N*-phenylanthranilic acid. The chromium content of all specimens is expressed as mg. atoms Cr/g. moisture-free collagen.

Total N was determined by a Kjeldahl method.

RESULTS

Preliminary experiments demonstrated that a considerable diminution in chromium fixation resulted from acetylation of collagen. The measurements illustrated in Fig. 1 were made to determine whether this difference arose from a diminished combining capacity or from a slower rate of reaction. Collagen and two acetylated derivatives were allowed to react with 30%-basic chromic sulphate. The results show that the reaction is almost complete after 3 days and that acetylation does diminish the capacity of the collagen to fix chromium. In all subsequent experiments a standard reaction period of 72 hr. was adopted.

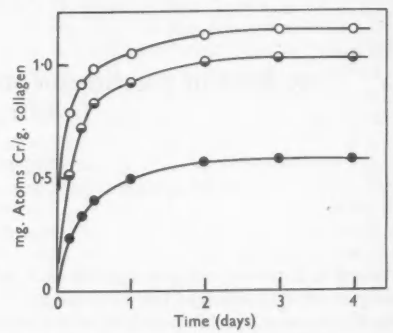

Fig. 1. Effect of time on fixation of chromium from a solution of the 30%-basic sulphate: ○, collagen; ◒, *N*-acetylcollagen (0·40 m-mole acetyl/g.); ●, fully acetylated collagen (1·73 m-moles acetyl/g.).

In Table 1 the analytical figures for several derivatives show that the loss of either *N*- or *O*-acetyl groups from acetylated collagen during chrome tanning is negligible. The behaviour of a number of collagen derivatives in chromic sulphate solutions of different basicities is shown in Fig. 2.

Fig. 3 illustrates how chromium fixation from solutions of both cationic and anionic chromium is influenced by the degree of acetylation of the collagen. Both *N*- and *O*-acetyl derivatives have been included in this figure, since chromium fixation seems to be linearly related to the total degree of acetylation, irrespective of whether amino or

Table 1. *Composition of collagen derivatives before and after reaction with 30 %-basic chromic sulphate for 3 days*

	Total N (%)	Cr		Acetyl	
		mg. Atoms/g. final product	mg. Atoms/g. collagen	m-Moles/g. final product	m-Moles/g. collagen
Collagen	18·0	—	—	—	—
Chromed collagen	16·0	1·10	1·24	—	—
N-Acetylcollagen	17·6	—	—	0·39	0·40
Chromed *N*-acetylcollagen	15·8	0·98	1·12	0·35	0·40
Fully acetylated collagen	16·6	—	—	1·60	1·73
Chromed fully acetylated collagen	15·4	0·67	0·78	1·39	1·63

hydroxyl groups are involved. Experimental points corresponding to not more than 0·40 m-mole total acetyl/g. collagen represent samples containing *N*-acetyl groups only; other points represent samples which are fully *N*-acetylated and contain some *O*-acetyl groups in addition. The scatter of the experimental points in this diagram is due in part to slow changes in the chromium solutions even after 2–3 months' ageing. The data of Fig. 3 were not all derived at the same time, and there appeared to be a very slow trend towards higher chromium fixation with age.

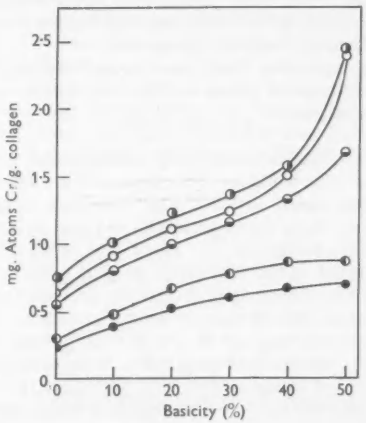

Fig. 2. Effect of basicity of chromic sulphate solution on chromium fixation by collagen and its derivatives: ○, collagen; ◑, denatured collagen; ◒, *N*-acetylcollagen; ●, fully acetylated collagen (1·73 m-moles acetyl/g.); ◐, fully acetylated denatured collagen (1·76 m-moles acetyl/g.).

The approximately linear relations demonstrated in Fig. 3 imply that the decrease in chromium fixation is a direct result of acetylation and is not a consequence of secondary physical changes in the substrate. This conclusion is strengthened by the data of Table 2. It is shown there that collagen containing no acetyl groups takes up a constant amount of chromium, whether it is the original material or whether it has been exposed to either acetic anhydride or acetic acid alone in the complete absence of water. Fully acetylated collagen, from which all *O*-acetyl groups have been removed by

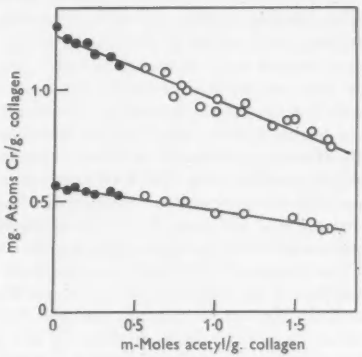

Fig. 3. Effect of degree of acetylation on fixation of chromium from 30 %-basic chromic sulphate (upper curve) and sodium oxalatochromiate (lower curve): ●, *N*-acetylated collagen; ○, collagen containing 0·40 m-mole *N*-acetyl/g. and further *O*-acetyl groups.

Table 2. *Chromium fixation by collagen containing no combined O-acetyl groups*

Pretreatment	Acetyl (m-moles/g. collagen)	Cr (mg. atoms/g. collagen)
None	—	1·24
Dried at 103°, soaked in acetic anhydride 3 days, extracted with acetone	—	1·26
Dried, soaked in acetic acid 3 days, extracted with acetone	—	1·20
Soaked in 0·1 N-NaOH, brought to pH 4 with acetic acid, washed 1 week	—	1·22
Alkaline de-acetylation fully acetylated collagen, thorough washing	0·41	1·18

treatment with alkali and thorough washing, still contains its full complement of *N*-acetyl radicals. Its chromium-binding capacity lies between that of the original collagen and that of *N*-acetylcollagen prepared by the standard method.

DISCUSSION

N-Acetylation. That the free amino groups of collagen might be involved in the chrome-tanning reaction was suggested by Thomas & Kelly (1926), who found that deaminated hide powder showed a greatly diminished chromium fixation from a 52 %-basic solution. Proceeding by a different method, Bowes & Kenten (1949) have reported similar findings, so that the effect of deamination is well established. Nevertheless, it is well known that side reactions occur when collagen is treated with nitrous acid, and some confirmatory evidence of the role of the amino groups is desirable. This is provided by the results given here for *N*-acetylated collagen.

It was found that *N*-acetylcollagen, prepared by the alkaline method, bound almost as much chromium as the original protein. However, this result was misleading, since raw collagen treated with a strong solution of sodium acetate and thoroughly washed was found to have enhanced chromium-binding capacity. On the other hand, as is shown in Fig. 2 and Table 2, treatment of collagen with either acetic anhydride or acetic acid in the absence of water, followed by extraction with acetone, leaves its capacity for chromium fixation unchanged. For that reason, all the *N*-acetyl derivatives used here were prepared by the action of a 5 % solution of acetic anhydride in acetic acid on the dry protein. The data reported in Table 1 and the slope of the upper curve in Fig. 3 both show that blocking free amino groups by acetylation decreases the amount of chromium fixed from a moderately basic chromic sulphate solution to the extent of about one atom $Cr/3NH_2$ groups. Only at 50 % basicity (Fig. 2) does the ratio depart widely from this figure. It is much less than the diminution reported by Thomas & Kelly (1926), suggesting that the effect of nitrous acid treatment on chromium fixation may not be confined to simple removal of amino groups. Indeed, in the light of our observations on *O*-acetylation, it would appear that simple deamination, in which amino groups are replaced by an equivalent number of hydroxyl radicals, should have no effect on chromium fixation. Bowes & Kenten (1949) found decreases in chromium uptake of as much as two atoms Cr/NH_2 group, but only at pH values above 4, where the chromium complex is in a very high state of aggregation and tends to be precipitated.

O-Acetylation. Since we have at present no method of acetylating the hydroxyl groups of a protein without simultaneously substituting the amino groups, the effect of blocking the former is best studied on collagen which is already completely *N*-acetylated. The presentation is simplified by the observation, already mentioned, that there is no significant difference between *N*- and *O*-acetylation in their effect on chromium fixation.

It is clear from the results shown in Figs. 1 and 3 that introduction of *O*-acetyl groups into collagen also diminishes its power to fix chromium, no matter how long the derivative is left in contact with the chromium solution. Denatured collagen, which takes up rather more chromium than native collagen under the same conditions, exhibits a similar loss of affinity upon complete acetylation. The full combining capacity of collagen is almost entirely restored if the *O*-acetyl groups are removed by dilute alkali followed by prolonged washing with water. Moreover, collagen samples which have been exposed, in the complete absence of water, to the action of either acetic anhydride or acetic acid alone, followed by thorough washing with acetone, and which contain no combined acetyl groups, have their combining capacity unimpaired. All these observations, together with the regular form of Fig. 3, leave little doubt that the decrease in chromium fixation is directly related to the blocking of hydroxyl groups in the protein.

The regression line drawn through the upper set of experimental points in Fig. 3 is represented by the equation:

$$y = 1{\cdot}19 - 0{\cdot}29x,$$

where x = m-moles acetyl/g. collagen and y = mg. atoms Cr/g. collagen. This means that, when the tanning agent is 30 %-basic chromic sulphate, between three and four amino or hydroxyl groups must be acetylated to block the entry of one chromium atom. Reference to Fig. 2 shows that this value is dependent on basicity and lies between one and four groups/Cr atom, increasing fairly regularly throughout the range 0–50 % basicity.

The course of the curves in Fig. 2 can be explained in terms of amino-acid content of collagen (Bowes & Kenten, 1948) by assuming that the primary reaction is between the polynuclear chromic complex and carboxyl, but that the product is stable only after ring formation has been achieved by the co-ordination of a second protein group at some point of the complex. The groups available for co-ordination are amino, guanidino, carboxyl or hydroxyl, whose relative abundance in collagen is approximately 1:1·3:1·9:4·4. In a highly acetylated collagen, with half of these groups inactivated, some carboxyl radicals will be too far distant from another group to permit co-ordination, and these carboxyl radicals will be unable to form stable compounds with chromium. Thus, at low basicities, we find acetylated collagen taking up far less than one

atom Cr/carboxyl group. With increasing basicity the size of the chromic complex increases, so that the carboxyl groups which can form stable compounds will now be associated with several chromium atoms. The more isolated groups remain ineffective, and we see that the ratio of fixed chromium to total carboxyl approaches unity. The absence of an upward inflexion from the curve for highly acetylated collagen may imply that the largest chromic complexes cannot be firmly held without the help of more than one stabilizing group. When all the amino and nearly all the hydroxyl groups have been acetylated, stabilization of the bound chromium is dependent entirely on the carboxyl and, perhaps, guanidino groups.

Normal collagen, on the other hand, contains about 2·8 amino or hydroxyl groups for every free carboxyl, and there is a much higher probability that every chromic complex reacting with carboxyl will also be able to co-ordinate with one or other of the former. More chromium will be fixed even from solutions of low basicity, and at high basicities the rapidly growing polynuclear complexes will come within the range of additional co-ordinating groups and will be firmly held. This double effect of large particle size in the chromic salt coupled with greater availability of amino and hydroxyl groups can be expected to cause the sharp upward sweep of the curves in Fig. 2.

The fact that several groups must be acetylated to block the entry of one chromium atom does not, of course, imply that all are attached to that atom. It is purely a probability effect resulting from the fact that all groups can be expected to be equally susceptible to acetylation but that only some of them are placed near carboxyl groups. If it is assumed that the amino and hydroxyl groups of collagen are randomly distributed, that all are equally effective in co-ordinating chromium and that all groups of the same kind are equally easily acetylated, then, at least at moderate basicities, the fixation of chromium should be linearly related to the degree of acetylation, as in Fig. 3. The lower curve of this figure shows that similar considerations apply to reactions with anionic chromium complexes.

SUMMARY

1. Acetylation of either free amino or free hydroxyl groups of collagen causes a marked diminution of its ability to take up either cationic or anionic chromium complexes from solution.

2. For chromic sulphate solutions, this effect has been studied over a wide range of basicities and for differing degrees of acetylation. It is most pronounced at high basicity and increases almost linearly with the degree of acetylation.

3. The experimental results suggest that, while the initial reaction between the chromic complex and collagen occurs at the carboxyl groups of the latter, the co-ordination of amino or hydroxyl radicals is essential to the formation of a stable compound.

4. In the basicity range normally employed in industrial chrome tanning, approximately one collagen amino or hydroxyl group in every three appears to be involved in the reaction.

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The Influence of Energy Intake on Protein Metabolism

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Although it is well known that protein metabolism is influenced by energy intake, the underlying biochemical mechanism remains obscure. We thought that it might be profitable to study animals under circumstances in which changes in energy intake fail to affect protein metabolism and to compare them with animals under conditions in which the normal relationship holds. Examination of the literature suggested that rats receiving low and high levels of protein intake provide the necessary contrast. Several reports indicate that reduction of the intake of a protein-free diet may not alter the nitrogen balance of rats (Mitchell, 1924; Treichler & Mitchell, 1941; Vars & Gurd, 1947), at least until intake represents less than half the voluntary consumption (Swanson, 1951). On the

other hand, addition of carbohydrate or fat to diets containing adequate amounts of protein results in an improvement in the nitrogen balance of the rat (Forbes, Bratzler, Thacker & Marcy, 1939; Forbes & Swift, 1944; Lathe & Peters, 1949).

In using these observations as a basis for study, it seemed desirable to establish the range of energy intake over which protein metabolism is affected at adequate levels of protein intake and at the same time the range over which the nitrogen balance is unaffected when the animal is receiving a protein-free diet. We have therefore carried out studies of the nitrogen balance and of the protein content of the liver of rats receiving diets rich in protein or deficient in protein, in combination with various levels of energy intake.

METHODS

The influence of energy intake on nitrogen balance and on the protein content of the liver was studied in four series of experiments. Two were carried out using diets containing protein and two with diets free from protein, and at each of these levels of protein intake the variations in energy intake were produced in one series of animals by changes in dietary carbohydrate and in the other series by changes in dietary fat. Two separate experiments were carried out under each of these four dietary conditions; as the results were similar, they have been combined in Exps. 1–4. In addition, a confirmatory experiment (no. 5) was carried out in which only the liver was studied.

Animals. Male albino rats about 5 months old were used. After fasting the stock overnight, sufficient rats weighing between 230 and 270 g. were selected for a single experiment. These were then distributed between the various experimental groups according to the randomized block technique (Snedecor, 1946). This statistical procedure permits one to reduce the effect of differences in initial body weight as a factor in the analysis of the results. The mean initial weights of the rats in Exps. 1–5 were 251, 255, 251, 253 and 258 g., respectively. Their body surface areas were computed from the formula $S = 12{\cdot}54 + W^{0{\cdot}60}$, where S is the surface area and W is the body weight (Lee, 1929).

Diets and general management. The rats were housed under thermostatic conditions (23–25°) in individual metabolism cages. Food was given twice daily in heavy ointment jars and was moistened with water to prevent scattering. At 9 a.m., 2 g. of a vitamin-mineral-roughage mixture (Munro, 1949) were given to all animals. At 5 p.m., rats on the protein-containing diet each received 2·8 g. of casein (Glaxo), 1·0 g. of potato starch, 1·0 g. of glucose and 0·5 g. of fat; in the case of the animals on the protein-free diet, the casein was replaced by an equivalent amount of carbohydrate (half starch and half glucose). These basic diets provided daily about 800–900 kg.cal./sq.m. of body surface area and carbohydrate or fat was added to them to bring the energy intake up to any desired level. The source of additional energy was incorporated in the morning meal, in order to ensure that we were dealing with the effect of energy intake *per se* and not with the interaction effect occurring when protein and carbohydrate are fed at the same meal (Munro, 1949).

At first all rats received an energy intake of approximately 1200 kg.cal./sq.m./day for a period of 7 days, in order that they might become accustomed to the diet. This level of energy intake was obtained by adding 3·5 g. of glucose (Exps. 1, 3 and 5) or 1·5 ml. of olive oil (Exps. 2 and 4) to the appropriate basal diets. During this preliminary period many of the rats on the protein-containing diet showed a slight loss of weight. However, comparison of rats losing weight with those gaining weight during the 7-day period does not suggest any influence of these changes in body weight upon the subsequent response of protein metabolism to variations in energy intake. As might be anticipated, there was a considerable loss of weight during the preliminary feeding period when the diet was free from protein. At the end of this period the energy intake of different animals was altered by addition to, or subtraction from, the diet of carbohydrate or fat fed in the morning meal. In this way different energy levels were provided under otherwise identical conditions. Because of the preliminary week of training to the diet, there was no difficulty in persuading animals to consume diets providing up to 1700 kg.cal./sq.m. of body surface area. Each rat was maintained for 4 days at the new level of energy intake. During this period urine and faeces were collected in Exps. 1–4 by the procedure described by Cuthbertson, McGirr & Robertson (1939), the faeces being marked with ferric oxide. At the end of this period the rats were killed by exsanguination under ether anaesthesia and the livers taken for determinations of protein, nucleic acids and phospholipins. In Exp. 2, the other viscera (stomach, intestines, heart, lungs, kidneys, testes and bladder) were also removed and analysed for nitrogen.

Analytical procedures. Nitrogen analyses on food, urine, faeces and tissues were carried out by a semi-micro-Kjeldahl method, using metallic Hg as the catalyst. The adequacy of the procedure was established by comparison with results obtained using the micromethod of Hiller, Plazin & Van Slyke (1948), which has been thoroughly checked by these authors and by comparison with results obtained on a food mixture with micro-Dumas determinations of N on the same sample.

The livers were analysed for protein, nucleic acids and phospholipins by a slight modification of the Schmidt-Thannhauser (1945) procedure. Each liver was weighed on a chilled watchglass and coarsely minced with scissors. About 0·5 g. of this was accurately weighed into 5 ml. of ice-cold 10% (w/v) A.R. trichloroacetic acid (TCA), ground with a glass rod and centrifuged. The precipitated material was washed twice with 10% TCA and then successively extracted with 5 ml. portions of 80% (w/v) ethanol, absolute ethanol, thrice with an ethanol-$CHCl_3$ mixture (3:1) at 75° for 0·5 hr. and finally with ether. The extracts were combined and the P content determined by the Allen (1940) procedure (lipid P). The residue, containing the nucleic acids and protein, was digested for 18 hr. in N-NaOH at 37°. A portion of the digest was neutralized and TCA added to give a final concentration of 10% (w/v). This precipitated the deoxyribonucleic acid; the precipitate was washed twice with 5% TCA and the supernatant and washings were combined for P estimation (ribonucleic acid P). The precipitate was dissolved in N-NaOH and the P content estimated (deoxyribonucleic acid P). Finally, the N content of the alkaline digest was determined as described above; from this the protein N was obtained by subtracting the contribution of

the nucleic acids to the N contained in the alkaline digest (ribonucleic acid P + deoxyribonucleic acid P × 1·69). The results of the ribonucleic acid and phospholipin analyses will be reported separately.

Statistical analyses. The relationship between energy intake and N balance or liver protein on the different diets was obtained by calculating the regression coefficients, using the analysis of variance technique to determine the significance of the results (Snedecor, 1946). In no instance did the regression lines deviate significantly from linearity.

RESULTS

The first four experiments show (Tables 1 and 2) that there was a linear relationship between energy intake and change in body weight. It is noteworthy that the influence of energy intake on body weight was greater in the two experiments with protein-containing diets than in the two experiments in which the diets were free from protein. This difference, which is statistically significant in the case of carbohydrate but not of fat, is presumably due to the considerable effect of energy intake on nitrogen balance when the diet contains protein, resulting in changes in body protein as well as body fat.

The influence of energy intake on nitrogen balance was found to be dependent on protein intake. In the case of additions of carbohydrate to the diet (Fig. 1), nitrogen balance was affected in a strictly linear fashion by the increments in energy intake when the

Table 1. *Changes in body weight and nitrogen output caused by alterations in energy intake over a 4-day period*

(Groups of four rats in Exps. 1, 3 and 4; groups of five in Exp. 2.)

Exp. no.	Diet	Source of energy variation	Daily energy intake (kg.cal.) Intake/rat	Intake/sq.m. body surface area*	Body weight change (g.)	N intake and ouput during the 4-day period: Intake (mg.)	Urine (mg.)	Faeces (mg.)
1	Protein-containing	Carbohydrate	28·2	850	−13·1	1425	1510	141
			37·5	1100	− 6·0	1425	1419	149
			47·0	1370	+ 1·4	1425	1271	168
			56·3	1660	+ 8·4	1425	1161	164
2	Protein-containing	Fat	28·2	830	− 9·8	1495	1476	124
			42·1	1230	− 0·7	1495	1425	119
			56·1	1610	+ 5·2	1495	1270	145
3	Protein-free	Carbohydrate	29·3	900	−18·5	13	304	117
			38·6	1180	−16·6	13	249	132
			48·1	1450	− 9·5	13	240	127
			56·7	1710	− 6·5	13	227	141
4	Protein-free	Fat	29·3	920	−13·1	13	186	130
			38·3	1190	− 9·0	13	197	130
			47·3	1460	− 7·1	13	214	135
			56·3	1730	+ 0·5	13	194	152

* Surface area calculated from mean body weight during the 4-day period according to the equation of Lee (1929).

Table 2. *Statistical analysis of the effect of changes in energy intake on body weight, nitrogen balance and liver protein*

(The original data are given in Table 1 and Figs. 1–3. The regression coefficients represent the change produced over a 4-day period by an increment in energy intake of 1000 kg.cal./sq.m. of body surface area.)

Exp. no.	Diet	Source of energy variation	Regression coefficients: Body weight (g.)	N balance (mg.)	Liver-protein N (mg.)
1	Protein-containing	Carbohydrate	+26·6*	+410*	+33·0*
2	Protein-containing	Fat	+19·3*	+237*	+46·1*
3	Protein-free	Carbohydrate	+15·7*	+69†	−11·4‡
4	Protein-free	Fat	+15·8*	−41‡	−4·1‡

The probability that the regression coefficient is different from zero is shown as follows:

* Statistically highly significant ($P < 0·01$).

† Although the linear regression coefficient is statistically significant ($P = 0·05 - 0·01$), the regression coefficient based on log. energy intake is more highly significant ($P < 0·01$).

‡ Not statistically significant ($P > 0·05$).

The regression coefficients in Exps. 1 and 2 are significantly different from those obtained in Exps. 3 and 4, except in the case of changes in body weight caused by addition of fat to the diets.

animals were receiving adequate amounts of protein. On the other hand, the same additions of carbohydrate had a much smaller effect on nitrogen balance when the diet was devoid of protein. Moreover, on the latter diet there was a closer relationship between nitrogen balance and the logarithm of energy intake than between nitrogen balance and energy intake itself (Table 2). This suggests a curvilinear relationship, as shown in Fig. 1, and implies that additions of carbohydrate to a protein-free diet become progressively less effective in influencing nitrogen balance as energy intake increases. In the case of additions of fat to the basal diets (Fig. 2), there was again a linear effect on nitrogen balance at adequate levels of protein intake. Fat added to the protein-free diet caused no significant change in N balance. Thus we find a similar positive influence when energy in the form of carbohydrate or fat is added to diets adequate in protein, whereas in the case of diets free from protein there is a failure of carbohydrate to influence nitrogen balance above energy intakes of about 1200 kg.cal./sq.m. and a failure of fat over the whole range studied. Comparison of the regression coefficients (Table 2) shows that the influence of protein level is highly significant in the case of both carbohydrate and fat. These changes in nitrogen balance are essentially due to alteration in urinary output; in all four experiments there was a slight tendency for faecal nitrogen output to rise with increasing energy intake (Table 1).

The effect of energy intake on the total amount of protein in the liver is also significantly influenced by protein intake (Fig. 3 and Table 2). At adequate levels of protein intake (Exps. 1 and 2), the amount per liver was positively influenced by increments in energy intake, whether produced by addition of carbohydrate or of fat. In the case of protein-free diets, on the other hand, there was a slight though not significant tendency for the amount of protein in the liver to decline as energy intake rose (Exps. 3 and 4). These results have been confirmed in Exp. 5, in which animals at adequate and at low levels of protein intake were studied simultaneously. Addition of carbohydrate to these diets to bring the energy intake up from about 850 to 1600 kg.cal./sq.m. resulted in an increase in liver protein on the high-protein diet and a fall in liver protein on the protein-free diet (Table 3). The difference in behaviour at the two levels of protein intake was again statistically highly significant.

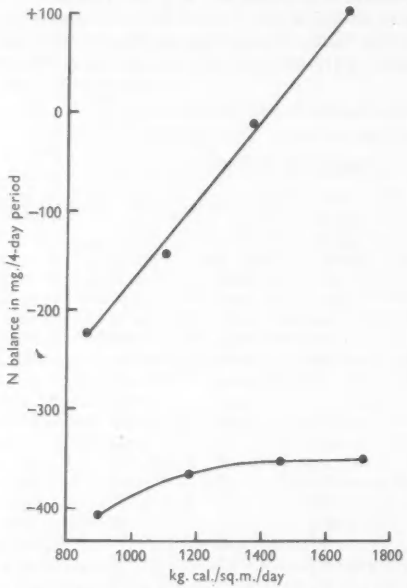

Fig. 1. The effect on nitrogen balance of changes in energy intake produced by alterations in dietary carbohydrate. Upper regression line, protein-containing diet (Exp. 1). Lower line (drawn freehand through points), protein-free diet (Exp. 3). Each point is the mean result obtained with four rats.

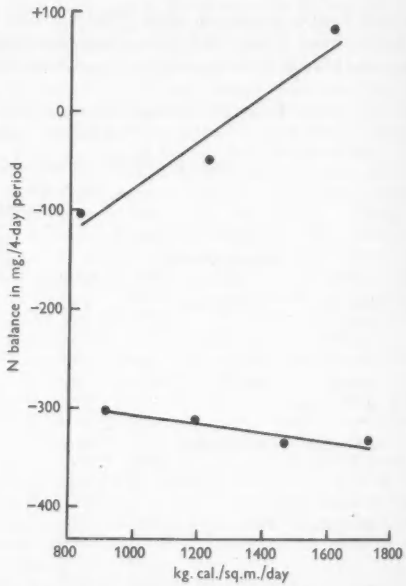

Fig. 2. The effect on nitrogen balance of changes in energy intake produced by alterations in dietary fat. Upper regression line, protein-containing diet (Exp. 2). Lower regression line, protein-free diet (Exp. 4). Each point is the mean result obtained with five rats (Exp. 2) or four rats (Exp. 4).

Exp. 2 provides some information about the partition within the body of changes in nitrogen balance brought about by alterations in the energy content of protein-containing diets. In addition to determinations of liver protein (Fig. 3), the total nitrogen content of the liver and of the other viscera was estimated. The data show (Table 4) that the

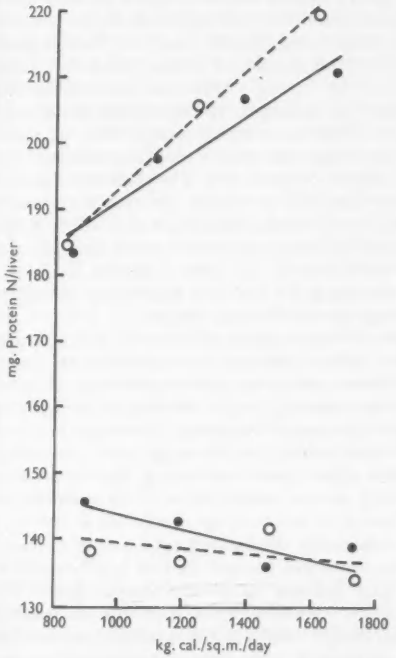

Fig. 3. Changes in liver-protein nitrogen produced by variations in energy intake from carbohydrate (●—●) or from fat (○---○). The upper two curves represent the regression lines obtained when the diet contained protein (Exps. 1 and 2). The lower two regression lines were obtained when the diet was free from protein (Exps. 3 and 4). Each point is the mean result obtained with four or five rats.

effect of energy intake on the total nitrogen content of the liver did not account for more than a quarter of the change occurring in nitrogen balance. Energy intake did not have a significant effect on the nitrogen content of the other viscera and it may be concluded that the major part of the change in nitrogen balance occurred through alterations in the amount of nitrogen in the carcass.

DISCUSSION

Exps. 1 and 2 illustrate the relationship between nitrogen balance and energy intake which obtains when the diet provides adequate amounts of protein. In these experiments energy intake was varied from sub-maintenance levels up to levels which caused appreciable increments in body weight and a positive nitrogen balance. Over this range the relationship of nitrogen balance to energy intake was a linear one without evidence of a break at or near nitrogen equilibrium (Figs. 1 and 2). Protein metabolism is thus in a state of dynamic equilibrium with energy intake, even when the animal is in nitrogen equilibrium, and any variation in energy level will lead to a corresponding alteration in nitrogen balance. Experiments on human subjects reported by Rubner (1903) and Neumann (1919) are compatible with our findings. Each of these authors studied a subject on a sub-maintenance diet to which was added increasing amounts of fat or carbohydrate. These additions caused a progressive improvement in nitrogen balance through equilibrium to a considerable nitrogen retention. Inspection of their data suggests that the relationship of nitrogen

Table 3. *The response of liver protein to changes in energy intake (carbohydrate) at different levels of protein intake*

(Exp. 5, ten rats per group. Analysis of variance demonstrates a significantly different response to changes in energy intake at the two levels of protein intake ($P<0.01$ for interaction). The regression coefficients represent the change produced by an increment in energy intake of 1000 kg.cal./sq.m. of body surface area. Both coefficients differ very significantly from zero and from one another ($P<0.01$).)

Diet	Daily energy intake (kg.cal./sq.m.)	Liver-protein N per rat (mg.)	Regression coefficients (mg.)
Protein-containing	820	191	+28·4
	1570	213	
Protein-free	880	142	−12·3
	1650	133	

Table 4. *The effect of adding fat to a protein-containing diet on the nitrogen content of the liver and other viscera, compared with the changes produced in the nitrogen balance of the whole rat*

(The regression coefficients represent the change produced by an increment of 1000 kg.cal./sq.m. body surface area. Exp. 2, five rats per group.)

Daily energy intake (kg.cal./sq.m.)	N balance per 4-day period (mg.)	Total liver N per rat (mg.)	Total viscera N per rat (mg.)
830	−105·0	240·9	460·9
1230	− 49·5	271·9	480·1
1610	+ 80·3	287·7	471·9
Regression coefficients	+237*	+60·3*	+14·5†

* Statistically highly significant ($P<0.01$).
† Not statistically significant ($P>0.05$).

balance to caloric intake was approximately linear over the range studied. Rosenthal & Allison (1951) describe experiments on dogs in which successive reductions in caloric intake caused a decline in nitrogen balance from positive at high-energy levels to negative at low-energy intakes. It is apparent from their data, however, that quite large changes in energy intake had little effect on nitrogen balance when the animals were near equilibrium. There is no obvious reason for this difference in behaviour of the dog from that of man and the rat.

Exps. 3 and 4 show that the relationship between energy intake and nitrogen balance is altered when protein is omitted from the diet. When carbohydrate intake was changed on a protein-free diet, nitrogen balance was little affected until energy intake fell below 1200 kg.cal./sq.m. of body surface area (Fig. 1). In the case of changes produced by varying fat intake, no significant alteration in nitrogen balance occurred over the whole range of caloric intake studied (Fig. 2). The difference in response to energy in the form of carbohydrate and energy in the form of fat may be related to the composition of the diet given prior to varying the energy intake. In the case of Exp. 3, the energy intake during the preparatory period was made up to 1200 kg.cal./sq.m. by adding carbohydrate to the basal protein-free diet, in order that the lowest energy intakes might be produced by removing this carbohydrate. In Exp. 4, fat was added to the basal diet during the preparatory period, for a similar reason. The fat content of the preparatory diet may have influenced protein metabolism during subsequent undernutrition. Samuels, Gilmore & Reinecke (1948) have observed that nitrogen output during starvation is reduced when the preceding diet contains a high proportion of fat, and Swanson (1951) has noted that restricting the intake of a protein-free diet has a less serious effect on nitrogen balance when the diet contains a high proportion of fat.

Essentially the same picture as that obtained from nitrogen balance studies was obtained from the study of liver protein in these experiments (Fig. 3 and Table 3). Liver protein varied in amount with energy intake in the case of the protein-containing diets, in agreement with Campbell & Kosterlitz (1948) who used the non-glycogen non-lipid solids as an estimate of liver protein, but increments in the energy content of the protein-free diets failed to raise the amount of liver protein. It will be noted in Exp. 3 that there was no change in the amount of liver protein corresponding to the slight improvement in nitrogen balance when carbohydrate was added to the protein-free diet.

These experiments on the relationship between energy intake and protein metabolism may at first sight seem most readily explicable on the hypothesis that the amount of dietary protein used for energy-yielding purposes is inversely proportional to the amount of energy provided from other dietary sources. Thus a decreasing energy intake would result in more and more dietary protein being utilized for supplying energy and less for protein synthesis. If this were so, one would expect the addition of a given amount of protein to the diet to be less effective in causing protein deposition in the body when energy intake was low, since a greater proportion of the added protein would be utilized to provide energy. However, studies on dogs (Allison & Anderson, 1945; Allison, Anderson & Seeley, 1946) have demonstrated that for a given increase in protein intake the same change occurs in nitrogen balance over a considerable range of energy intakes. A similar interpretation can be placed on the observation by Campbell & Kosterlitz (1948) that the amount of protein (non-glycogen, non-lipid solids) in the liver of the rat is related to protein intake by the same regression coefficient at different levels of energy intake.

An alternative explanation of our data is in terms of the factors affecting protein synthesis. Protein synthesis depends on the supply both of amino-acids and of energy, either of which can be a limiting factor in the rate of synthesis. In the case of protein-free diets, energy intake may influence nitrogen balance up to a certain point (e.g. 1200 kg.cal./sq.m. of body surface area in Exp. 3) by promoting re-utilization of amino-acids circulating in the blood, but eventually the limited nature of this source of amino-acids will prevent further improvements in nitrogen balance as energy intake rises. When protein is included in the diet this limitation no longer obtains and protein synthesis proceeds at a rate dependent on the level of energy-yielding metabolites in the tissues. In Exp. 3 nitrogen balance was influenced by changes in energy intake up to 1200 kg.cal./sq.m., but over this range the protein content of the liver did not alter significantly. This indicates that some tissues are less sensitive than the liver to the limitation imposed by a protein-free diet.

A final point which emerges from these experiments is the bodily distribution of changes in nitrogen balance produced by varying the energy content of a diet containing protein (Table 4). Changes in the total nitrogen of the liver accounted for about a quarter of the alteration in nitrogen balance. Since the nitrogen content of the other viscera was not significantly altered, the main change must have taken place in the carcass, i.e. muscle, skin, bone and adipose tissue. Of these, muscle seems the most likely tissue to be involved in such metabolic changes. It is unlikely that the protein of adipose tissue was significantly altered by deposition or removal of fat, since similar changes in

the fat content of the body must have occurred when the energy content of the protein-free diet was altered. Although the changes in liver nitrogen account for only a small proportion of the total change in nitrogen balance, the effect on liver composition is considerable. If we take the nitrogen content of the liver at an energy intake of 1200 kg.cal./sq.m. as our reference standard, then a change in energy intake of 1000 kg.cal./sq.m. caused an alteration of 23 % in the nitrogen contained in the liver. The nitrogen in the carcass of a 250 g. rat amounts to about 6 g., so that the change in nitrogen balance per 1000 kg.cal. not accounted for by the liver would represent only 3 % of the carcass nitrogen. The relative magnitudes of the change in the liver and in the carcass resemble those found by Addis, Poo & Lew (1936) when they altered the protein content of the rat diet. Calculations based on their data show that the liver contained 53 % more protein after 5 days of a high-protein diet than on the 10th day of a protein-free diet, whereas the carcass protein had changed by only 4 %. The ratio of the change in liver protein content to carcass protein content is of the same order of magnitude as the relationship found by us for the effect of changes in energy intake, and this strengthens our belief that the influence of protein intake and of energy intake on protein metabolism operates through a common mechanism, namely their effect on protein synthesis.

SUMMARY

1. Studies of nitrogen balance and the protein content of the liver were made on rats receiving diets either rich in protein or deficient in protein, in combination with various levels of energy intake (from about 850 to 1700 kg.cal./sq.m. of body surface area) which were obtained by adding carbohydrate or fat to standard basal diets.

2. When the diet provided adequate amounts of protein, increments in energy intake, produced by adding either carbohydrate or fat to a sub-maintenance diet, caused a linear improvement in nitrogen balance. Protein metabolism must thus be in a state of dynamic equilibrium with energy intake over the range studied. The total amount of protein contained in the liver also responded to increasing energy intake in a linear fashion. Changes in energy intake caused a smaller percentage change in the N content of the carcass than in the N content of liver.

3. When the diet contained no protein, addition of fat to bring energy intake up from 900 to 1700 kg.cal./sq.m. failed to influence nitrogen balance. Addition of carbohydrate produced some improvement up to 1200 kg.cal./sq.m. but not thereafter. In neither case was the amount of protein in the liver significantly altered.

4. It is suggested that, when the supply of amino-acids circulating to the tissues comes solely from endogenous sources, this becomes a limiting factor in the rate of protein synthesis at quite low levels of energy intake. When the diet supplies adequate amounts of protein this limitation no longer obtains.

We wish to thank Prof. J. W. Cook, F.R.S., for arranging to have micro-Dumas nitrogen determinations carried out by the technical staff of the Chemistry Department, and Dr W. C. Hutchison of this department for instructing us in the method of liver analysis by the modified Schmidt-Thannhauser procedure. One of us (H. N. M.) wishes to acknowledge with gratitude the receipt of a grant for expenses from the Medical Research Council.

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The Influence of Energy Intake on Ribonucleic Acid Metabolism

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In a preceding paper (Munro & Naismith, 1953), we described the influence of energy intake on the protein metabolism of the rat. The caloric value of the diet was varied from about 850 to about 1700 kg.cal./sq.m. of body surface area and it was observed that, when the diet provided adequate amounts of protein, this change in energy intake was accompanied by a considerable alteration in nitrogen balance and in the amount of protein in the liver, high energy intakes being associated with positive nitrogen balances and larger amounts of liver protein. On the other hand, when the diet contained no protein, increments in energy intake had little or no influence on nitrogen balance, and, if anything, caused a reduction in the amount of protein in the liver. It was thought that the conditions provided in these experiments might be suitable for studying the nature of the alleged relationship between ribonucleic acid (RNA) and protein synthesis (Brachet, 1950; Caspersson, 1950). We have therefore investigated the influence of energy intake on the total amount of RNA in the liver and on the rate of incorporation of ^{32}P into liver RNA, both when the diet contained adequate amounts of protein and when it was free from protein. A preliminary account of these experiments has already appeared (Munro, Naismith & Wikramanayake, 1952).

METHODS

Total ribonucleic acid in the liver

The livers studied came from the rats used in the investigation of the relationship between energy intake and protein metabolism, described previously (Munro & Naismith, 1953). Full details of their selection and feeding and methods of analysis employed are given there. Briefly, male rats of about 250 g. body weight were given either a diet containing adequate amounts of protein or a protein-free diet, fed for the first week at a level of 1200 kg.cal./sq.m. of body surface area. Then for 4 days different rats received different energy intakes, produced by varying the amount of carbohydrate or fat in the diet. At the end of this period the rats were killed by exsanguination and the livers removed. The livers were then submitted to a modified Schmidt-Thannhauser separation procedure to give the amounts of protein, RNA, deoxyribonucleic acid and phospholipin present.

The first four experiments deal with the addition of carbohydrate or fat to protein-containing and to protein-free diets. These were carried out as separate experiments. In a fifth (confirmatory) experiment, the effect of additions of carbohydrate to protein-containing and protein-free diets were studied simultaneously. In addition, a further confirmatory experiment was performed in which the effect of adding fat to a protein-free diet was compared with the effect of adding carbohydrate. This last experiment is not described in the paper on protein metabolism.

The amounts of ribonucleic acid found are expressed as RNA/liver. Since the rats were initially of the same size, consistent differences in RNA/liver can be ascribed to the diets used. Statistical analyses were carried out as before. The regression coefficients presented represent the change produced by an alteration in energy intake of 1000 kg.cal./sq.m. of body surface area. There were no significant deviations from linearity of regression.

Incorporation of ^{32}P *into ribonucleic acid*

Experiments were carried out on a different series of animals from those described above. Male albino rats were used, but the choice was not restricted to those of 250 g. body weight. Within each experiment, animals of closely similar weight were selected, but the mean initial weight varied in different experiments from about 180 to 230 g.

Except for two experiments (Table 6 and Fig. 3), the feeding arrangements were similar to those used in studying the influence of energy intake on total RNAP/liver. The energy intake was adjusted to 1200 kg.cal./sq.m. of body surface area for 1 week, and was then varied so that some rats received a low intake of energy whilst others received a high intake, the other dietary constituents being kept constant. Unless otherwise stated, these changes in energy intake were produced by varying the amount of carbohydrate in the diet. The rats were maintained at these different energy levels for 4 days. On the last day they were injected intramuscularly with ^{32}P as inorganic phosphate (10 μc./100 g. of body weight) and were killed by exsanguination under ether anaesthesia after 4, 8 or 24 hr., in most experiments at 24 hr. The liver was quickly perfused with 0·9% saline and then subjected to the slightly modified Schmidt-Thannhauser procedure described by Davidson & Smellie (1952). In this way an alkaline digest was obtained containing, amongst other things, deoxyribonucleic acid and ribonucleotides. The deoxyribonucleic acid was precipitated by bringing the digest to pH 3 with 60% (w/v) perchloric acid, and removed by centrifugation. The precipitate was washed twice with N-perchloric acid and the supernatant and washings combined for ionophoretic separation of the ribonucleotides. This was carried out by the procedure described by Davidson & Smellie (1952). In most cases, separation was only carried out over a 7 hr. period with a potential gradient of 15 V./cm. of filter paper. This is sufficient to provide a considerable separation of contaminant phosphorus compounds from the ribonucleotides, but

does not allow elution of the individual nucleotides separately. In some cases, we also submitted the digest to ionophoretic separation over a period of 18 hr. at a potential gradient of 11 V./cm. and obtained separation of individual nucleotides. After elution, P estimations and determinations of radioactivity were performed as described by Davidson, Frazer & Hutchison (1951). The radioactivity of the inorganic phosphate of the liver was obtained by precipitation of $Mg(NH_4)PO_4$, as described by Davidson *et al.* (1951); in view of the criticism of some forms of the $Mg(NH_4)PO_4$ precipitation procedure by Ennor & Rosenberg (1952), it should be noted that no carrier phosphate was used. In a few instances the specific activity of the acid-soluble organic phosphorus fraction (S_1 fraction of Davidson *et al.* 1951) was determined.

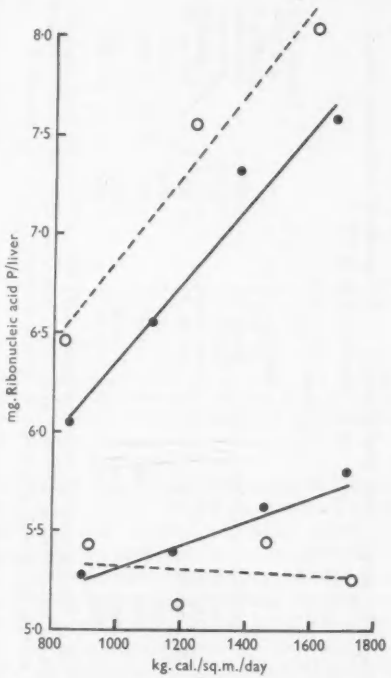

Fig. 1. Changes in liver ribonucleic acid produced by variations in intake of carbohydrate (●—●) or fat (○ - - - ○). The upper two curves represent the regression lines obtained when the diet contained protein. The lower two regression lines were obtained when the diet was free from protein. Each point is the mean result obtained with four or five rats.

The results have been expressed as specific activities (counts/min./100 μg. P) and also as relative specific activities (specific activity of organic phosphate P divided by the specific activity of inorganic P of liver multiplied by 100). The data have been analysed by recognized statistical procedures (Snedecor, 1946).

RESULTS

Energy intake and the quantity of ribonucleic acid in the liver

When the diet contained adequate amounts of protein, the amount of RNA in the liver varied considerably with energy intake (Fig. 1). This occurred whether carbohydrate or fat was the variable energy source in the diet, the regression coefficient being +1·96 mg. RNAP/1000 kg.cal. in the case of carbohydrate and +2·01 mg. RNAP/1000 kg.cal. in the case of fat; both coefficients were highly significant ($P<0·01$). In the case of the protein-free diet, additions of carbohydrate caused a smaller, though still significant, rise in the amount of RNAP/liver (+0·60 mg./1000 kg.cal.; $P<0·01$) but additions of fat failed to do so (−0·07 mg. RNAP/1000 kg.cal.; $P>0·05$). The regression coefficients obtained in the case of the protein-free diets are both significantly less than those obtained with the protein-containing diets ($P<0·01$). Comparison of the addition of carbohydrate to the protein-free diet with the addition of fat also suggests a difference in action, though here the level of statistical significance ($P=0·05-0·02$) is not conclusive. These conclusions are based on the first four experiments which were not carried out at the same time. Confirmatory experiments were therefore performed in which different dietary groups were studied simultaneously. The first of these (Table 1) demonstrates that addition of carbohydrate to a protein-containing diet

Table 1. *The effect of changes in energy intake (carbohydrate) at different levels of protein intake on the ribonucleic acid content of the liver*

(Groups of ten rats.)

Diet	Daily energy intake (kg.cal./sq.m.)	Total RNAP/liver (mg.)
Protein-containing	820	6·42
	1570	7·51
Protein-free	880	5·26
	1650	5·48

Analysis of variance indicates a highly significant ($P<0·01$) effect of protein intake and of energy intake on the quantities of RNAP and a very significant difference in the influence of energy intake at the two levels of protein intake ($P<0·01$ for interaction).

had a significantly greater influence on the RNA content of the liver than its addition to a protein-free diet. In a second experiment (Table 2) we compared the effect of adding carbohydrate to a protein-free diet with the effect of adding fat, and were unable to demonstrate a significant difference in their influence on the amount of RNA/liver. Thus the essential point established is that the amount of

RNA in the liver is much more sensitive to changes in energy intake when the diet contains protein than when it is devoid of protein.

Table 2. *Comparison of the effect on liver ribonucleic acid of adding carbohydrate or fat to a protein-free diet*

(Groups of five rats.)

Source of energy variation	Daily energy intake (kg.cal./sq.m.)	Total Liver RNAP/rat (mg.)
Carbohydrate	880	5·34
	1650	5·29
Fat	880	5·28
	1660	5·49

Analysis of variance does not reveal any significant difference between the action of carbohydrate and of fat.

Energy intake and ^{32}P incorporation into ribonucleic acid

The influence of increasing energy intakes on the incorporation of ^{32}P into the RNA of liver is illustrated in Table 3. The animals were killed 24 hr. after injection of the isotope. The phosphorus of RNA is eventually derived from liver inorganic phosphate, and the specific activity of RNAP will therefore be influenced by the specific activity of the inorganic phosphate; variations in this factor can be allowed for by expressing the results relative to the specific activity of liver inorganic phosphate, i.e. relative specific activity. In the case of rats receiving protein in the diet, the relative specific activity tends to fall as energy intake rises, whereas on the protein-free diet there is an appreciable rise in relative specific activity. An alternative method of correcting the specific activity of RNAP for differences in the activity of inorganic phosphate is the statistical procedure of covariance analysis (Snedecor, 1946); this allows one to adjust the observed specific activities of RNAP to give the values which would have been obtained if the specific activity of liver inorganic phosphate had been the same for all animals. The correction is made solely from evidence of the relationship between the specific activities of RNAP and inorganic phosphate provided by the experiment itself. In the present case, such an analysis (Table 3, column 7) confirms completely the picture obtained from the study of relative specific activity.

The experiment just described is representative of a series of observations on rats killed 24 hr. after injection of ^{32}P. Over the whole series, thirty-three animals received the protein-free diet and eighteen were given the protein-containing diet, energy intake ranging from 600 to 1900 kg.cal./sq.m. of body surface area. The change in relative specific activity induced by alterations in energy intake over this range is expressed by the regression coefficients/1000 kg.cal./sq.m., namely +10·3 for the

Table 3. *The effect of changes in energy intake (carbohydrate) on the uptake of radioactive phosphorus by liver ribonucleic acid 24 hr. after injection of ^{32}P*

(Groups of four rats.)

Diet	Mean initial body weight (g.)	Daily energy intake (kg.cal./sq.m.)	Specific activity of RNAP (counts/min./100 μg. P)	Specific activity of liver inorganic phosphate (counts/min./100 μg. P)	Relative specific activity of RNAP	Specific activity of RNAP adjusted by covariance analysis (counts/min./100 μg. P)	Total RNAP/liver (mg.)	Total radioactivity of liver RNAP, adjusted by covariance analysis (counts/min.)
Protein-containing	185	810	580	1527	37·9	693	3·25	22 300
	186	1715	665	1896	35·8	646	4·22	27 600
Protein-free	193	820	761	2004	38·2	680	3·18	21 300
	189	1790	839	1747	48·4	857	3·70	31 600

Analysis of variance shows that the effect of changes in energy intake was significantly different ($P<0·05$) for interaction at the two levels of protein intake for the relative specific activity of RNAP and for the specific activity adjusted by covariance analysis. The total radioactivity/liver showed a highly significant response to energy intake ($P<0·01$), but this effect was not significantly greater for the protein-free diet ($P>0·05$ for interaction).

protein-free diet and +1·2 for the protein-containing diet. If we take the relative specific activity at 1200 kg.cal. as our reference standard, these regression coefficients represent a change of +26·5 and +3·4% respectively, in relative specific activity. The change in rate of incorporation is significant for the protein-free diet ($P<0·01$) but not for the protein-containing diet.

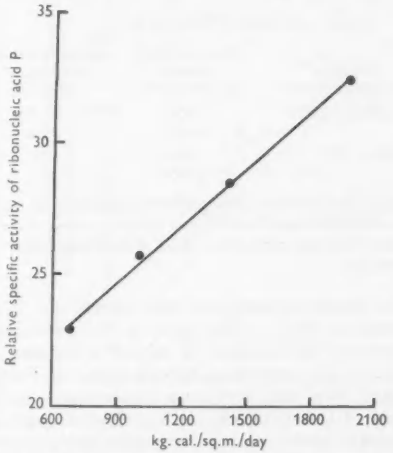

Fig. 2. The effect of adding carbohydrate to a protein-free diet on the relative specific activity of ribonucleic acid phosphorus. The rats were injected with ^{32}P as orthophosphate 24 hr. before killing. Each point is the mean of three observations.

In order to confirm the magnitude of the change found in the case of the protein-free diet, a further experiment was carried out in which carbohydrate was added to a protein-free diet to give four levels of energy intake. The results obtained (Fig. 2) leave no doubt of the linear relationship between energy intake and relative specific activity of RNAP. From the calculation of the regression coefficient, it was ascertained that an increment of 1000 kg.cal./sq.m. of surface area resulted in a 27·8% alteration in relative specific activity, again using the value at 1200 kg.cal./sq.m. as our reference standard ($P<0·05$).

The observations so far described concern only the values obtained 24 hr. after injection of labelled phosphorus. The same picture has, however, been observed with animals killed at shorter time intervals. Rats prepared in the same way as above were killed at 4 and 8 hr., as well as at 24 hr., after ^{32}P was administered. In order to reduce the amount of work involved, we studied only three dietary groups, namely animals receiving the protein-free diet at low and at high levels of energy intake, and animals

Table 4. *The effect of changes in energy intake (carbohydrate) on the uptake of ^{32}P by liver ribonucleic acid at 4, 8 and 24 hr. after injection of labelled P*

(Each figure is the mean of observations made on four rats.)

Time after ^{32}P injection (hr.)	Type of diet	Mean daily energy intake (kg.cal./sq.m.)	Mean initial body weight (g.)	Specific activity of inorganic P of liver (counts/min./100 μg. P)	Relative specific activity of acid-soluble organic P	Relative specific activity of nucleotides			
						Whole RNAP*	Adenylic and cytidylic acids	Guanylic acid	Uridylic acid
4	Protein-free	870	179	4860	84	7·5	7·6	5·2	8·7
	Protein-free	1880	179	4714	85	9·8	9·4	8·2	11·3
	Protein-containing	1840	181	3638	—	4·4	—	—	—
8	Protein-free	950	177	3063	102	17·0	16·9	14·1	18·5
	Protein-free	1850	182	2419	110	19·2	19·1	17·5	21·3
	Protein-containing	1840	186	2234	93	13·1	12·6	10·2	16·3
24	Protein-free	840	186	1769	105	34·1	33·1	32·5	34·6
	Protein-free	1890	173	2237	104	54·2	51·6	51·6	54·1
	Protein-containing	1725	184	1808	100	32·1	31·2	28·6	34·2

* Short run ionophoresis, with nucleotides eluted together.

receiving the protein-containing diet at the higher plane of energy intake. These three groups provide all the necessary contrasts (cf. Table 3). In all three groups the specific activity of liver inorganic phosphate showed a similar trend with time (Table 4). The relative specific activities of RNAP from animals receiving the protein-free diet at the higher plane of energy intake were the greatest at all time intervals after injection. In addition, this experiment indicates the behaviour of individual ribonucleotides obtained by prolonging the period of ionophoresis. Guanylic and uridylic acids were completely separated, but it was found necessary to elute adenylic and cytidylic acids together. It is apparent (Table 4) that, under the same conditions of diet and time after isotope injection, the various nucleotides incorporate ^{32}P to essentially the same extent. Each nucleotide shows a higher incorporation rate when obtained from animals on the protein-free diet at the higher level of energy intake. This would appear to eliminate the possibility that contaminants play a significant part in accounting for the differences in phosphorus incorporation into RNA, since they could hardly affect all nucleotide fractions similarly. A further feature of this experiment is the behaviour of the acid-soluble organic phosphates of the liver (Table 4). The specific activity of this fraction is not significantly altered by the nature of the diet at any of the time intervals after injection of the labelled phosphate. This suggests that the changes displayed by RNA phosphorus in relation to diet are not shared by phosphorylation in general.

The experiments so far described refer to changes in energy intake produced by alterations in dietary carbohydrate. The effect of adding fat to a protein-free diet has also been determined. In these experiments one series of rats was maintained at an energy intake of 1200 kg.cal./sq.m. by adding carbohydrate to a protein-free diet and another series by adding fat; after a week at this level, different energy intakes were obtained in each series by varying the amount of carbohydrate or fat fed. Table 5 shows that the addition of fat was at least as effective as the addition of carbohydrate in stimulating the rate of incorporation of ^{32}P into RNA. An additional experiment with fat has confirmed this picture.

Table 5. *Comparison of the effect on ^{32}P uptake by liver ribonucleic acid of adding fat or carbohydrate to a protein-free diet*

(Groups of three rats.)

Source of energy variation	Daily energy intake (kg.cal./sq.m.)	Relative specific activity of RNAP
Carbohydrate	1000	25·7
	1920	32·4
Fat	980	23·2
	1900	30·5

Analysis of variance shows that the influence of energy intake is significant ($P<0·05$) but that the action of carbohydrate does not differ from that of fat (interaction not significant).

Two final experiments were performed in an attempt to throw some light on the mechanism underlying the changes in RNAP synthesis observed in the preceding experiments; they differ slightly from the foregoing experiments in the details of the diets fed. In the first of these additional experiments, rats were fed either a protein-containing or a protein-free diet at a high level of energy intake for a week, and then each was injected with labelled phosphate. Thereafter, half of each dietary group were given glucose *ad lib.* and the rest were fasted. All animals were killed 24 hr. after injection. The data show (Table 6) that the rate of ^{32}P incorporation was determined by the previous diet and not by the immediate ingestion of glucose. Thus the group on the protein-free diet did not lose their higher incorporation rate when fasted 24 hr., and the high-protein group did not exhibit an increased incorporation rate when fed for 1 day exclusively on glucose. This picture is amplified by

Table 6. *The effect of glucose administration during a 24 hr. period following high- and low-protein diets on total ribonucleic acid/liver and the rate of ^{32}P incorporation into ribonucleic acid*

(Groups of four rats.)

Diet	Initial body weight (g.)	Daily energy intake on diet (kg.cal./sq.m.)	Food during final 24 hr.	Total RNAP/liver (mg.)	Relative specific activity of RNAP
Protein-containing	226	1680	Glucose	5·09	39·9
	230	1680	None	4·65	36·7
Protein-free	233	1705	Glucose	4·22	48·2
	230	1725	None	4·56	53·2

Analysis of variance shows that the amount of RNAP in the liver is significantly affected ($P<0·05$) by the administration of glucose during the final 24 hr., when the preceding diet contained protein. There is a highly significant difference ($P<0·01$) in relative specific activity at the two protein levels, but no significant effect of glucose at either protein level.

the final experiment (Fig. 3). In this case, all animals were first fed a diet providing an adequate amount of protein and energy (1700 kg.cal./sq.m.).

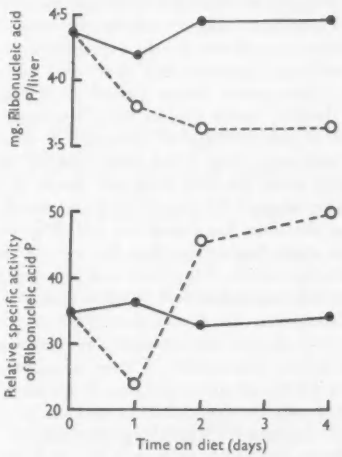

Fig. 3. The influence of protein intake on the amount of ribonucleic acid in the liver (upper two curves) and its uptake of radioactive phosphorus (lower two curves). After a week on an adequate diet, the rats were given either a diet containing protein (●—●) or a similar diet free from protein (○---○), both diets being fed at a level of 1700 kg.cal./sq.m. body surface/day. The animals were injected with ^{32}P as orthophosphate 24 hr. before killing. Each point is the mean of observations on three rats.

After they had been conditioned to this diet for a week, half the animals were given a protein-free diet of equicaloric value, carbohydrate being substituted for protein. Thereafter, groups of three rats from each diet were killed daily, having been injected 24 hr. previously with labelled phosphate. The transfer to the diet lacking protein caused an immediate reduction in the RNAP/liver but did not induce an immediate rise in the rate of ^{32}P incorporation. In fact there was first a fall, followed by the expected higher level from the second day onwards. The rats which continued to receive the protein-containing diet (controls) showed a constant amount of RNAP/liver and no significant alteration in the rate of ^{32}P incorporation. According to Student's 't' test (Snedecor, 1946), both the initial reduction in ^{32}P incorporation rate on the first day of the protein-free diet and the subsequent increase in rate differ significantly ($P < 0{\cdot}01$) from the rate of incorporation by the control rats killed on the same day.

DISCUSSION

The above experiments indicate that energy intake influences the metabolism of RNA. In order to compare the magnitude of the alterations in amount and in rate of incorporation of ^{32}P caused by variations in energy supply, we have calculated the change produced by an increment of 1000 kg.cal./sq.m. of body surface area and have expressed it as a percentage of the amount or the incorporation rate obtaining at an energy intake of 1200 kg.cal./sq.m. In Table 7, all experiments involving a change in energy intake from carbohydrate have been set out in this way (experiments with fat are fewer, but show the same general trends). With regard to changes in total RNA/liver, we have not only the results obtained by the orthodox Schmidt-Thannhauser procedure, but also more accurate estimates of the amount of RNA obtained in the course of the radioactivity experiments by ionophoretic separation of the ribonucleotides. Table 7 shows that, when the diet

Table 7. *Comparison of the changes in total ribonucleic acid/liver and ^{32}P uptake by ribonucleic acid when energy intake from carbohydrate is increased*

(Regression coefficients indicate the change produced by adding 1000 kg.cal./sq.m. body surface area to the diet, and have been expressed as a percentage of the amount of RNAP or the relative specific activity of RNAP corresponding to an energy intake of 1200 kg.cal. Figures in brackets are the number of animals in each experiment.)

			Regression coefficients	
			Total RNAP/liver	Relative specific activity of RNAP
Diet	Data recorded in	Method of RNA isolation	(%)	(%)
Protein-containing	Fig. 1	Schmidt-Thannhauser	+29·2 (16)	—
	Table 1	Schmidt-Thannhauser	+20·8 (20)	—
	—	Ionophoresis	+28·6 (24)	+ 3·4 (16)
Protein-free	Fig. 1	Schmidt-Thannhauser	+10·9 (16)	—
	Tables 1 and 2*	Schmidt-Thannhauser	+ 4·4 (24)	—
	—	Ionophoresis	+ 8·1 (57)	+26·5 (33)
	Fig. 2	Ionophoresis	—	+27·8 (12)

* The animals shown in Table 1 as receiving a protein-free diet include most of those given in Table 2 (carbohydrate added).

provides adequate amounts of protein, an increase in energy intake of 1000 kg.cal. causes a rise of some 20–30 % in total RNAP/liver, without significantly altering the proportion of P atoms, replaced in a given time. With a protein-free diet, the total amount of RNAP is only slightly increased (about 5–10 %) when the intake of energy is raised by 1000 kg.cal., but the rate of replacement of P atoms is augmented by about 25 %. It is thus apparent that, at each level of protein intake, the total number of P atoms incorporated into RNA is increased by raising the intake of energy, in one case by an increase in total RNAP/liver without a change in the percentage of P atoms incorporated in a given time, in the other case by an increase in incorporation rate with a smaller change in total RNAP/liver. It is clear from Table 7 that the magnitude of the change in incorporation rate on the protein-free diet is fully adequate to compensate for the lack of alteration in total amount. This can also be demonstrated more directly by calculations based on the data given in Table 3. By multiplying the specific activity of the RNAP by total RNAP/liver, we obtain a measure of the total amount of ^{32}P incorporated per liver. This has been done in Table 3 (last column), the figures being adjusted by covariance analysis for differences in the specific activity of the inorganic phosphate of the liver. It will be seen that at each level of protein intake, a rise in energy intake is accompanied by an increased absolute incorporation of ^{32}P. The effect of energy intake on ^{32}P incorporation is not significantly different at the two levels of protein intake.

It is thus clear that two dietary factors influence the course of RNA metabolism in the liver. Protein intake plays a dominant role in determining the amount of RNA in the liver, a finding in agreement with that of Campbell & Kosterlitz (1950, 1952), whereas energy intake determines the absolute rate of phosphorus incorporation. This explains why the unrestricted feeding of a protein-free diet results in a reduction in liver RNA accompanied by a compensatory rise in the turnover of phosphorus in the remaining molecules (Fig. 3). Campbell & Kosterlitz (1948, 1952) were the first to draw attention to this phenomenon, although they were unaware of its explanation.

The observation that energy intake determines the absolute rate of ^{32}P incorporation into RNA raises the question of whether this reflects real differences in the rate of RNA synthesis or whether the phosphorus is incorporated by an exchange reaction. Decisive evidence can only be obtained by studying incorporation of isotopes into other parts of the nucleotide structure, but it should be pointed out that, in view of the commonly accepted structure of RNA, it seems unlikely that most of the P atoms can be exchanged with the medium except by molecular resynthesis. Even if we accept the data as representing the behaviour of the whole RNA molecule, the significance of the findings remains obscure. In this connexion two of our experiments present suggestive features. Changes in energy intake do not immediately affect the rate of ^{32}P incorporation, as shown by the experiment in which rats receiving a protein-free diet with abundant energy intake were either fasted or fed glucose shortly before death (Table 6). Furthermore, in contrast to the findings of Campbell & Kosterlitz (1948) with unpurified RNA preparations, removal of protein from the diet does not result in an increased uptake of ^{32}P by RNA until the level of RNA in the liver has ceased to fall (Fig. 3). This suggests that, during the first day of the protein-free diet, the needs of the liver cell are met by the considerable breakdown of RNA attached to protein. Thereafter, the requirements of the cell can only be provided by increasing the percentage turnover of RNA. The nature of these requirements is a matter for future investigation. It seems unlikely that they can be explained in relation to protein synthesis, since increments in energy intake do not increase the amount of protein in the liver when the animals are receiving a protein-free diet (Munro & Naismith, 1953), whereas RNA synthesis is increased under these circumstances. This point can, however, only be finally decided by simultaneous study of the rates of RNA and protein synthesis on protein-free diets. Finally, there is the possibility that RNA serves as a source of nucleotides for coenzyme synthesis, in which case it would not be unnatural to find that the requirements of the cell for RNA were proportional to energy intake.

SUMMARY

1. A study has been made of the effect of variations in energy intake on the amount of ribonucleic acid (RNA) in the rat liver and on the uptake of labelled phosphorus by RNA.

2. When the basal (sub-maintenance) diet contained protein, addition of energy in the form of either carbohydrate or fat resulted in a considerable increase in total RNA per liver; when the diet lacked protein, an increase in energy intake caused only minimal changes in the amount of RNA.

3. The uptake of ^{32}P by RNA, as measured by relative specific activity, behaved in the opposite way. Uptake was unaffected by variations in energy intake when the diet contained protein, but it was considerably stimulated by addition of energy to the protein-free diet.

4. These results indicate that the absolute rate of incorporation of phosphorus into RNA is dependent on energy intake. At each level of protein intake, the total number of phosphorus atoms incor-

porated into RNA is increased by raising energy intake, in one case by an increase in the total RNA per liver without a change in the percentage of phosphorus atoms incorporated in a given time, in the other case by an increase in percentage incorporation rate accompanied by a smaller change in total RNA per liver.

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Biochemical Changes Occurring During the Germination of Bacterial Spores

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Spores of laboratory strains of *Bacillus subtilis* and *Bacillus megatherium*, incubated with L-alanine and glucose respectively, remain viable, but rapidly lose their resistance to heat (Hills, 1950; Powell, 1951). They also become less refractile and more permeable to stains (Powell, 1951). These changes indicate the first stages of spore germination and are accompanied by a decrease in cell dry weight and an increase in total and amino nitrogen of the suspending medium. It appears that excretion is one of the first changes associated with the germination process, whether this occurs in a medium containing only the specific germination stimulant or in a nutrient tryptic digest broth (T.M.B.), or spontaneously in water suspension as a result of prolonged heat activation. This excretion process and the identification of the excreted solids will be described in detail.

ORGANISMS AND METHODS

Most of the work was done with spores of the laboratory strain of *B. megatherium*. These were obtained by growth on meat extract peptone agar containing 0·2% Lab Lemco, 1% peptone, 0·5% NaCl, and 0·2% glucose (Tarr, 1933). They were reaped after 2 days' growth at 37° and washed at least five times with water. During reaping and the first three washings, suspensions were cooled to 0°, since it had been previously found that some incompletely washed suspensions germinated spontaneously at room temperature. Spores of this organism need glucose specifically for germination and, in some cases, a short preliminary heat treatment, i.e. 30 min. at 60° (Powell, 1951). It has been recently found that if a prolonged heat treatment is given, i.e. 2 hr. at 60°, and the suspension is then centrifuged and resuspended in water, spontaneous germination takes place. Within an hour, more than 90% of the spores lose their heat resistance and stain densely, but are still viable. These changes occur in suspensions as thick as $1{\cdot}5 \times 10^{10}$ spores/ml. Germination exudates were obtained in this way, and also by incubating resting spores with 5 mM-glucose and 30 mM-phosphate buffer, pH 7·3. After 30–45 min. incubation the suspension was centrifuged, and the clear and colourless centrifugate removed for analysis.

A less complete investigation was made with spores of the laboratory *B. subtilis*. These were obtained after 3–4 days' growth at 37° on casein-yeast (CCY) agar (Gladstone & Fildes, 1940). They were washed five times with water, and had no tendency to germinate spontaneously during reaping or after heat treatment. In 30 mM-phosphate with 2 mM-L-alanine and 5 mM-glucose, germination was generally not more than 30–50% in suspensions of 5×10^9 spores/ml., and could only be improved by the addition of disproportionally high concentrations of L-alanine which then obscured subsequent analyses and amino-acid chromatograms. Germination was almost complete in suspensions

of 2×10^9 spores/ml. after 30–45 min. incubation with 2 mM-L-alanine and 5 mM-glucose in 30 mM-phosphate at pH 7·3. Analyses were made on centrifugates from these suspensions.

Total nitrogen. This was determined by the Dumas and the micro-Kjeldahl methods. In the latter method, the catalyst suggested by Chibnall, Rees & Williams (1943) was used.

Amino nitrogen. This was determined by the nitrous acid reaction (Peters & Van Slyke, 1932) and α-amino nitrogen by the method of Pope & Stevens (1939).

Paper chromatography. For the detection of amino-acids, this was carried out as described by Consden, Gordon & Martin (1944) and for purines and pyrimidines as described by Markham & Smith (1949).

Hexosamine. This was determined by the method of Elson & Morgan (1933) with the modification suggested by Immers & Vasseur (1950).

Calcium. This was determined by titration with permanganate after precipitation as oxalate (Kramer & Tisdall, 1921).

The Unicam spectrophotometer was used to measure ultraviolet absorption spectra.

RESULTS

Decrease in dry weight during germination

Excretion during germination of *B. megatherium* was assessed both by measuring decrease in cell dry weight and increase in dry weight of the suspending medium. This was centrifuged off and a sample heated in an oven at 103° for 24 hr. Cell dry weight was similarly determined before and after germination. In the latter case, the cells were washed with 0·9% sodium chloride before drying. Weight changes corresponding with complete germination were calculated after determining percentage germination from stained films. The results obtained are shown in Table 1.

The figures given in the last two columns are derived from the means of duplicate dry weights. The values for gain in weight of the medium are consistently higher than those for loss in weight of the cells. This can partly be accounted for by inadequate washing and retention of saline in the centrifuged cell mass. It is also possible that germination may involve depolymerization, i.e. hydrolytic, processes which might be expected to increase the cell dry weight to a small extent. It was interesting to find that, owing to this weight loss during germination, it was possible to separate germinated from resting spores by centrifugation. During germination, spores of *B. megatherium* became also appreciably darker in colour, so that when a partly germinated suspension was centrifuged, the separation of the cells into two layers could be distinctly seen.

Since germination of spore suspensions thicker than 2×10^9/ml. of *B. subtilis* was poor, even in T.M.B., it was not practicable to determine increase in dry weight of the medium, since this increase, with spore suspensions of 2×10^9/ml., is relatively low. Decrease in cell dry weight at spore concentrations of 2×10^9/ml. was determined both in T.M.B. and the alanine-glucose phosphate medium. It was found to be 26% in the meat digest and 35% in the chemically defined medium.

Excretion of nitrogen-containing compounds during germination

When a suspension of *B. megatherium* spores containing 5×10^9 spores/ml. was incubated for 45 min. with 5 mM-glucose in 30 mM-phosphate, 80% germination occurred. The total N (Kjeldahl) of the medium rose to 9·7 and the amino N to 4·5 mg./100 ml. In a similar experiment with spores of *B. subtilis* and the addition of 2 mM-L-alanine to the above medium, there was 38% germination in 60 min. The total N of the medium increased by 3·8 and the amino N by 2·0 mg./100 ml. Slightly greater nitrogen excretion was found with spores of *B. megatherium* when T.M.B. was the germination medium.

Excretion of ultraviolet-absorbing compounds during germination

Resting spores of *B. subtilis* and *B. megatherium* were suspended at 10^9 spores/ml. in 30 mM-phosphate buffer, pH 7·3, and broken up mechanically at 0° in a tissue disintegrator (Mickle, 1948) with Ballotini beads, size 12. After 45 min. shaking, very few intact cells could be found in a stained film. The disintegrated suspension was centrifuged at high

Table 1. *Decrease in dry weight of* Bacillus megatherium *spores during germination*

Medium (10 ml.)	No. of spores	Spore dry wt. (mg.)	Germination (%)	Change in dry weight for complete germination (mg./100 mg.) Cells	Medium
T.M.B.	$1{\cdot}08 \times 10^{11}$	105	90	−30	+33
	$1{\cdot}15 \times 10^{11}$	108	73	−27	+36
5 mM-Glucose, 30 mM-phosphate, pH 7·3	$1{\cdot}08 \times 10^{11}$	105	69	−29	+33
Distilled water (heat-activated germination)	$1{\cdot}15 \times 10^{11}$	108	95	−26 −25	+33

speed, the clear supernatant solution removed, and its ultraviolet absorption measured throughout the range 2000–3000 A. at 25 A. intervals. A characteristic absorption spectrum appeared with maxima at 2625, 2700 and 2775 A. Essentially the same spectrum was obtained when the media in which germination had occurred were similarly examined (Figs. 1 and 2). It seemed, therefore, that the ultraviolet-absorbing material present in resting spores had been excreted during germination. The identification of this material, thought at first to be a mixture of purines and pyrimidines, as dipicolinic acid, will be described in the following paper (Powell, 1953).

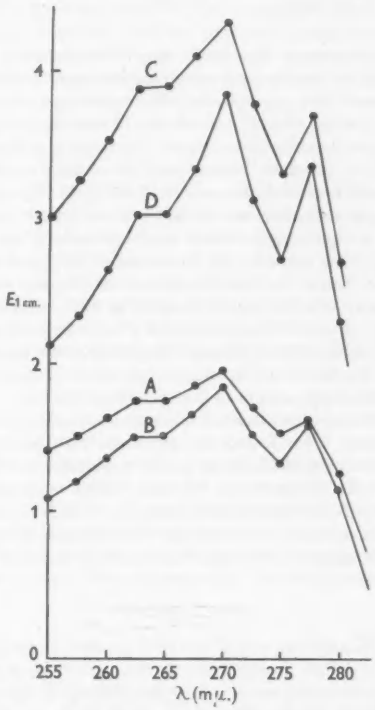

Fig. 1. Curve *A*, ultraviolet absorption of an extract from a disintegrated resting suspension of *B. subtilis* containing 10^9 spores/ml. Curve *B*, absorption of L-alanine phosphate medium after complete germination of *B. subtilis* has occurred at 10^9 spores/ml. Curve *C*, ultraviolet absorption of an extract from a disintegrated resting suspension of *B. megatherium* containing 10^9 spores/ml. Curve *D*, absorption of glucose phosphate medium after complete germination has occurred at 10^9 spores/ml.

Analysis of exudate from Bacillus megatherium *spores during heat-activated germination*

From the above results, it appears very likely that the excretory process associated with germination in both the organisms examined is essentially the same, whatever the medium in which it occurs.

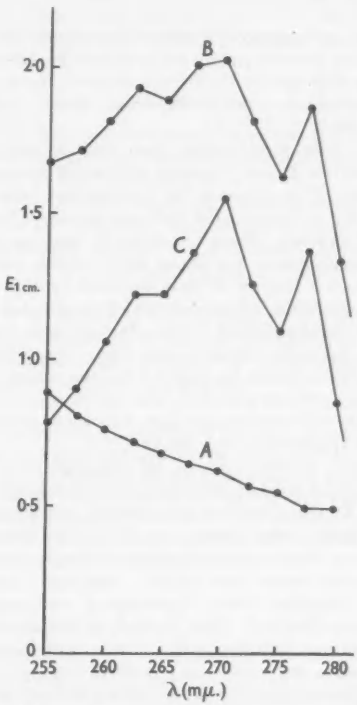

Fig. 2. Curve *A*, ultraviolet absorption of diluted meat digest medium. Curve *B*, absorption of meat digest medium after complete germination of *B. megatherium* has occurred at 4×10^8 spores/ml. Curve *C*, absorption of germination exudate derived by subtracting Curve *A* from curve *C*.

For more detailed investigation of the process, analyses were made of exudates from *B. megatherium* spores germinating spontaneously in distilled water after heat treatment. These exudates, prepared as described above, were filtered through sintered glass and freeze-dried. The resulting white feathery material had the composition C, 37·9; H, 4·9; N, 8·6 (Dumas); Ca, 9·5 %. It contained no phosphorus. There was good agreement between values obtained for nitrogen content of the crude exudate by the Dumas and the Kjeldahl techniques. This was surprising in view of a later finding that analysis

Table 2. *Analysis of freeze-dried exudate from heat-activated* Bacillus megatherium *spores*

	Nitrogen (g./100 g.)				
	Total	Ammonia N	Amino N	α-Amino N	Hexosamine (g./100 g.)	Sulphated ash (g./100 g.)
Unhydrolysed	8·48 (Kjeldahl) 8·60 (Dumas)	0·52	3·15	2·48	0	35·2
Hydrolysed	—	—	4·50	2·92	5·70	—

of the pure ultraviolet-absorbing material by the Kjeldahl method gave a value rather less than half of that obtained by the Dumas method. A summary of the analyses made on the crude exudate is given in Table 2.

The results indicated that amino-acids were present (see below), together with small amounts of peptide. The presence of hexosamine was first detected on amino-acid chromatograms of hydrolysed exudate. These compounds and ammonia nitrogen account for about 60% of the nitrogen present. A further 30% is supplied by the ultraviolet-absorbing compound, which constituted 50% of the total excreted solids. Purines and pyrimidines may be present, but their characteristic absorption would be masked by the other ultraviolet-absorbing material. The ash content of the exudate was remarkably high, and could pratically all be accounted for as calcium.

Paper chromatograms of unhydrolysed and hydrolysed exudate were run with phenol and collidine. In the unhydrolysed material, glutamic acid and glycine were present in the largest amounts, together with smaller quantities of alanine, leucine, isoleucine, serine and valine. Arginine, aspartic acid, histidine, lysine, methionine, and tyrosine were also detected. After hydrolysis, the amount of alanine and aspartic acid present had increased markedly, the threonine, αε-diaminopimelic acid and glucosamine appeared. Both glucosamine and αε-diaminopimelic acid appeared to be associated with a non-dialysable peptide fraction, which constituted approximately 20% of the total solid exudate (see below).

In an attempt to separate and identify the ultraviolet absorbing fraction, paper chromatograms were run in *tert.* butanol/hydrochloric acid and photographed using ultraviolet light (Markham & Smith, 1949). The absorbing material travelled as a single spot, and faster than any of the components of hydrolysed yeast nucleic acid.

Separation of the glucosamine-containing peptide from exudate of germinating Bacillus megatherium

A specimen of the crude freeze-dried exudate was dialysed in a cellophan sac at 0° against distilled water. The sac contents and the diffusate were freeze-dried. The diffusate contained only a trace of hexosamine. The freeze-dried dialysate was dissolved in water and precipitated with 2 vol. of ethanol. The precipitate was centrifuged, washed with 70% ethanol, dissolved in water, dialysed again and finally freeze-dried. The resulting material gave a positive biuret, and a weakly positive Molisch reaction. It contained 6·9% N (Kjeldahl). The glucosamine content after 20 hr. hydrolysis at 100° with 3N-hydrochloric acid in a sealed tube was 28%. This agreed with the value of 26% reducing sugar found by the Hagedorn & Jensen (1923) method, after 20 hr. hydrolysis at 100° with 0·5N-HCl. Paper chromatography on hydrolysates demonstrated the presence of glucosamine, aspartic and glutamic acids, serine, glycine, αε-diaminopimelic acid, alanine, valine and arginine.

Glucosamine attached to a non-dialysable peptide has also been found in exudates from spores of *B. subtilis* germinating in the L-alanine medium. A crude preparation of this peptide contained 5·25% N and 22·6% hexosamine. It also contained aspartic and αε-diaminopimelic acids, alanine, serine, glycine and small amounts of arginine and lysine.

DISCUSSION

It appears that one of the first processes occurring during spore germination is the excretion of solid material to the extent of approximately 30% of the spore dry weight. Resting spores have a remarkably high specific gravity of 1·46 (McIntosh & Selbie, 1937) and little or no metabolic activity (Keilin & Hartree, 1947; Spencer & Powell, 1952). This, considered together with their heat-resisting properties, suggests that their water content must be extremely low. So far, only one recorded attempt has been made to determine the water content of resting spores and to compare it with that of vegetative cells (Henry & Friedman, 1937). These authors measured the loss in weight which occurred when 'air-dried' cells were desiccated over calcium chloride and then heated at 105–110° for 4 days. They found that 'air-dried' vegetative cells and spores lost approximately the same amount of water, i.e. 16%, under these conditions. It is certain, however, and the above authors themselves point out, that 'air-dried' cells do not represent a satisfactory starting material, so that the value of these experiments seems somewhat doubtful.

We are attempting in this laboratory to measure the amount of water in resting spores of *B. subtilis* from a comparison of their wet and dry weights. Wet weight is calculated from measurements of spore density and volume. Dry weight is determined by heating a known number of spores at 104° for 2–3 days, followed by desiccation over phosphorus pentoxide *in vacuo* at 78°. Preliminary experiments strongly support the view that resting spores contain little or no water. It would seem that the germination process involves a sudden change in cell permeability followed by a simultaneous exchange of water from the medium for solids from the spore.

Of the solid materials excreted, the calcium, the ultraviolet-absorbing compound identified as dipicolinic acid (Powell, 1953), and the glucosamine peptide are perhaps the most interesting. It has been suggested that the high calcium content of spores may contribute in some way to their heat resistance, especially since thermophilic organisms contain substantially more calcium than mesophilic vegetative organisms (Curran, Brunstetter & Myers, 1943). The loss of calcium associated with loss of heat resistance supports this view. The role of dipicolinic acid in sporulation and development of heat resistance has still to be investigated. Preliminary experiments with spores of other members of the *Bacillus* group indicate the presence of dipicolinic acid in every case. The compound does not appear to be present in vegetative cells of this group.

The glucosamine-containing peptide appearing in the medium during germination is non-dialysable. It appears at a very early stage in the development of the spore, i.e. long before the emergence of the typical vegetative cell and the shedding of the spore coat (Harris & Powell, 1951). It may be derived from the spore integument when this undergoes the radical change in permeability associated with germination. It would be interesting to compare the peptides 'excreted' by these and other members of the *Bacillus* group with a view to the possibility of their possessing specific antigenic properties (Lamanna, 1940).

SUMMARY

1. The germination process in spores of *Bacillus megatherium* and *Bacillus subtilis* is accompanied by a 30% loss in cell dry weight.

2. The material excreted consists mainly of amino-acids, peptides, hexosamine attached to a non-dialysable peptide, and a substance absorbing strongly in the ultraviolet, with maxima at 2625, 2700, 2775 A.

3. It is suggested that resting spores contain little or no water, and that during germination there is a simultaneous exchange of water from the medium for solids from the spore.

The authors wish to thank Mr F. Belton for growing large spore crops, Dr R. B. Record for freeze-drying spore exudates, Mr S. Bailey and Mr N. Harkness for technical assistance, and Mr F. E. Charlton for a micro-analysis. A specimen of $\alpha\epsilon$-diaminopimelic acid was very kindly provided by Mrs Elizabeth Work. Permission to publish has been granted by the Chief Scientist, Ministry of Supply.

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Isolation of Dipicolinic Acid (Pyridine-2:6-dicarboxylic Acid) from Spores of *Bacillus megatherium*

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In a previous paper (Powell & Strange, 1953) the loss in dry weight of spores of *Bacillus megatherium* and *Bacillus subtilis* during germination was described, and accounted for by the excretion into the medium of amino-acids, peptides, glucosamine, calcium and a compound with a strong and characteristic ultraviolet absorption spectrum. In this paper the isolation and identification of the latter compound is described in detail.

MATERIALS AND METHODS

Lutidines (2:6- and 2:4-dimethylpyridines) were redistilled preparations from the compounds supplied by L. Light and Co.

Dipicolinic acid (pyridine-2:6-dicarboxylic acid) and lutidinic acid (pyridine-2:4-dicarboxylic acid) were prepared from the corresponding lutidines by oxidation with $KMnO_4$ in neutral solution (Hess & Wissing, 1915).

Quinolinic acid was recrystallized from the specimen supplied by L. Light and Co.

Dimethyl ester of dipicolinic acid. Dipicolinic acid (100 mg.) was dissolved in 20 ml. methanol saturated with dry HCl. After refluxing for 24 hr. the ester was precipitated by adding Na_2CO_3 solution. It was recrystallized from water. M.p. 122°.

Ultraviolet absorption spectra were measured with the Unicam spectrophotometer.

EXPERIMENTAL

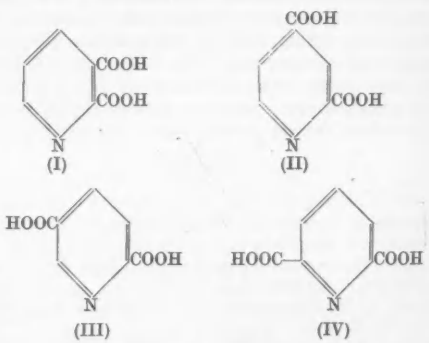
Isolation of the ultraviolet absorbing compound from exudates of germinating Bacillus megatherium *spores*

In preliminary attempts to isolate the ultraviolet-absorbing material, it was precipitated as an impure silver salt from media in which germination had occurred. A much simpler method of isolation was later discovered. The substance is acidic and occurs in exudates as a sparingly soluble calcium salt. Freeze-dried exudates were prepared from *B. megatherium* spores which had germinated spontaneously as a result of heat-activation (Powell & Strange, 1953). The resulting white feathery material was dissolved in the minimum amount of hot water. The calcium salt of the unknown acid crystallized out on cooling. After two further crystallizations from water, this salt was analysed, and found to contain C, 36·3; H, 2·7; N, 5·7; Ca, 15·8%. The free acid was readily obtained by acidifying a hot saturated solution of the calcium salt with HCl, and recrystallized from water. It crystallized in hair-like needles, m.p. 229° with decomposition. Analysis of the free acid gave C, 50·8; H, 3·1; N, 8·3%. On titration with dilute NaOH, one equivalent of alkali neutralized 81·5 g. of acid.

Identification of the ultraviolet-absorbing compound as dipicolinic acid

The first clue as to the nature of the compound appeared when the calcium salt was heated strongly in a tube. It decomposed with a marked smell of pyridine. The distillate was collected, and its ultraviolet absorption spectrum examined. It showed characteristic pyridine absorption, with maxima at 2450, 2500, 2575 and 2625 A.

This production of pyridine as a decomposition product, together with the analytical figures for the free acid and its calcium salt, indicated a pyridinedicarboxylic acid of empirical formula $C_7H_5O_4N$ (C, 50·2; H, 3·0; N, 8·4%) with a calcium salt $C_7H_3O_4NCa\ 2H_2O$ (C, 34·8; H, 2·9; N, 5·8; Ca, 16·6%). This was supported by the alkali-titration figure. The acid from spores gave a strong orange-yellow colour with $FeSO_4$ in aqueous solution, indicating that at least one carboxyl group is in the α position.



Of the four possible pyridine dicarboxylic acids with one carboxyl group in the α position, quinolinic acid (I) was eliminated after examination of its ultraviolet absorption spectrum. Moreover, quinolinic acid readily gave a fluorescein with resorcinol and H_2SO_4 (Ghosh, 1919) while the unknown acid did not. Lutidinic acid (II) gave only a faint colour with $FeSO_4$ and also had an ultraviolet absorption spectrum different from that of the unknown acid. Isocinchomeronic acid (III) was not examined, but seemed unlikely on the grounds of melting point and crystalline appearance. Finally, dipicolinic acid (IV) was prepared from 2:6-lutidine. Its ultraviolet absorption and that of its calcium salt were found to be identical with the corresponding compounds from spores (Figs. 1–3).

The dimethyl esters of dipicolinic acid and of the unknown acid were then prepared. Both substances melted at 122° and there was no depression of melting point on mixing.

It seems reasonably certain, therefore, that the ultraviolet-absorbing material which is present in resting spores of *B. megatherium* and *B. subtilis* and is excreted during germination is dipicolinic acid.

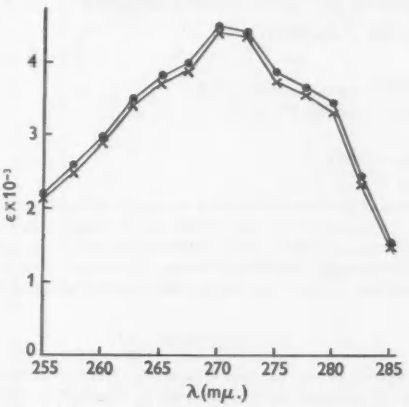


Fig. 1. Molecular extinction coefficients in 0·05 N-HCl. ●—●, dipicolinic acid; ×—×, pyridine dicarboxylic acid from spores.

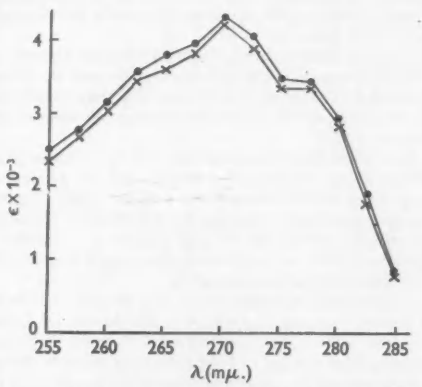


Fig. 2. Molecular extinction coefficients in 0·05 N-NaOH and 30 mM-phosphate, pH 7·3. ●—●, dipicolinic acid; ×—×, pyridine dicarboxylic acid from spores.

DISCUSSION

Dipicolinic acid has not previously been recognized as a constituent of living matter. Calcium dipicolinic constitutes 50% of the solids excreted by germinating spores of *B. megatherium* and, therefore, approximately 15% of the spore dry weight (Powell & Strange, 1953). Its physiological properties have yet to be determined. It was interesting to find that the calcium salt of dipicolinic acid is remarkable in its relatively high absorption at 2775 A. (Fig. 3) the ratio $\epsilon_{2700}/\epsilon_{2775}$ being 1:12. The corresponding ratios for the sodium, magnesium, barium, and aluminium salts lie in the range 1·20–1·26. It is possible, therefore, that the calcium salt has some difference in structure which may fit it for a special role in the formation and maintenance of the resting spore.

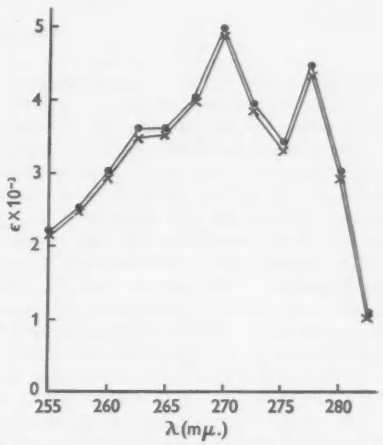


Fig. 3. Molecular extinction coefficients in 30 mM-phosphate, pH 7·3. ●—●, calcium dipicolinate; ×—×, calcium salt of pyridine dicarboxylic acid from spores.

Spore extracts from other members of the *Bacillus* group are now being examined and, so far, the characteristic dipicolinate absorption spectrum has been found in every case. It is hoped to extend these observations to the *Clostridium* group and also to observe the appearance of dipicolinic acid or possible precursors in cells during growth and sporulation.

SUMMARY

1. An acidic compound with strong and characteristic ultraviolet absorption (maxima at 2625, 2700 and 2775 A.) has been isolated, in the form of its calcium salt, from exudates of germinating spores of *Bacillus megatherium*.

2. The compound has been identified as dipicolinic acid.

I am greatly indebted to Mr L. H. Kent for very helpful advice and discussion. I also wish to thank Dr B. R. Record for freeze-drying, Mr F. E. Charlton for micro-analyses, and Mr S. Bailey for technical assistance. Permission to publish has been granted by the Chief Scientist, Ministry of Supply.

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The Separation of Steroid Alcohols by Chromatography of their Benzoates on Alumina

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The separation of steroids by chromatography on alumina has been practised by nearly all workers in this field during the last 15 years (for a review, see Reichstein & Shoppee, 1949). Steroids containing hydroxyl groups have commonly been subjected to chromatography as free alcohols or as acetates. Recently, in work on the steroids of pregnant mares' urine, we were faced with the problem of separating epimeric 20-hydroxysteroids. These are almost impossible to separate by chromatography as alcohols, and difficult to separate as acetates (for references, see p. 215). We therefore attempted the chromatography of the benzoates and found that pairs of 20-epimeric benzoates could be separated almost quantitatively in one chromatogram. Other examples of the chromatography of steroid benzoates in the literature are the separation of 17α- and 17$\alpha\beta$-hydroxy derivatives of *D*-homosteroids (Goldberg & Wydler, 1943 and subsequent papers by the same school), of the 5α:6α-epoxy- and 5β:6β-epoxy-cholestan-3β-ols (Barton & Miller, 1950) and of some steroid ketones lacking ring *D* (Cardwell, Cornforth, Duff, Holtermann & Robinson, 1951).

Since no systematic study of the chromatography of steroid benzoates had been made, we examined the behaviour of synthetic mixtures of a number of pairs of isomeric steroid benzoates of types likely to occur in work on natural products, especially urine. The results have shown that the chromatography of benzoates always offers at least as good a chance of separating isomers as does the chromatography of acetates, and sometimes a much better one. Turner & Voitle (1951) have independently discovered the value of benzoates as derivatives for the separation of 20-epimeric hydroxysteroids.

A preliminary account of this work has been given to the Biochemical Society (Brooks, Klyne & Miller, 1951). The application of the method to the separation of the non-ketonic β-hydroxy steroids of mares' urine has been described by Brooks, Klyne, Miller & Paterson (1952).

Nomenclature. This follows the proposals of the Ciba Foundation Conference (1951). Since this work has been concerned with the separation of 5-epimeric steroids, among others, it has seemed desirable to specify the configurations at $C_{(5)}$ explicitly in the names of all compounds—thus '5β-pregnane-3β:20β-diol'—to distinguish this isomer from 5α-pregnane-3β:20β-diol, although the name 'pregnane', according to the rules, implies the β-configuration at $C_{(5)}$.

EXPERIMENTAL

Melting points and optical rotations. Melting points and optical rotations were determined as described by Brooks *et al.* (1952). Melting points are all corrected. Optical rotations are all determined in $CHCl_3$ (*c*, 1–2%) at 20–25°; experimental error for $[\alpha]_D$ approx. $\pm 1°$. Samples for optical rotations and analyses were dried for 2 hr. *in vacuo* at 110° (except for compounds whose melting points were less than 140°, which were dried at 80°).

Analyses. These were by Weiler and Strauss, Oxford.

Starting materials. The hydroxysteroids used as starting materials for the preparation of benzoates and acetates were mostly gifts from colleagues to whom acknowledgements are made on p. 217.

Androstane-3β:17β-diol was prepared by reduction of the corresponding 17-ketone by $LiAlH_4$ (cf. preparation of pregn-5-ene-3β:20β-diol by Klyne & Miller, 1950). Pregn-5-ene-3β:20α-diol was obtained by reduction of the corresponding 20-ketone by Na and ethanol (cf. Wieland & Miescher, 1949) and separated from its 20β-epimer by chromatography of the benzoates.

Preparation of benzoates. These were prepared by the use of benzoyl chloride and pyridine on the alcohols (cf. Brooks *et al.* 1952). They were usually freed from traces of coloured impurities by passage in light petroleum-benzene through a short column of Al_2O_3. The physical properties of all the benzoates used and the analytical data for new compounds are summarized in Table 1. Most of the benzoates were recrystallized from $CHCl_3$:methanol (1:4, v/v). Exceptions are noted in Table 1. For comparison this table includes some data obtained by other workers on related compounds.

Chromatograms. These were carried out using Al_2O_3 (Savory & Moore Ltd.) of activity II–III (Brockmann & Schodder, 1941) and mixtures of light petroleum (b.p. 60–80°) and benzene of gradually increasing benzene content. All proportions of solvent mixtures are given as % (v/v). Standard proportions were steroid (1 g.), Al_2O_3 (60 g.), eluent (100 ml. fractions).

Most pairs of epimeric steroids were subjected to chromatography as acetates and as benzoates, using mixtures of about 15 mg. of each isomer. In a few cases the products of preparations were examined on a larger scale.

Table 1. *Properties of steroid benzoates*

Table 1. *Properties of steroid benzoates*

(Values for $[\alpha]_D$ were determined in $CHCl_3$ and have been rounded off to the nearest 1°; error ±1°. Analyses are given for new compounds only. Data determined by other workers are in italics.)

Compound	Molecular formula	M.p. (corr.)	Crystalline form	$[\alpha]_D$	$[M]_D$	Analyses: Found C (%)	Found H (%)	Required C (%)	Required H (%)	Notes
C_{19} steroids: monobenzoates										
3α-Hydroxy-5α-androstan-17-one	$C_{26}H_{34}O_3$	178°	Needles	+75°	+295°	—	—	—	—	(*a*)
3β-Hydroxy-5α-androstan-17-one		*216°*	Prisms	*+69°*	*+272°*	—	—	—	—	(*b*)
3α-Hydroxy-5β-androstan-17-one		164°	Needles	+98°	+387°	—	—	—	—	(*c*)
3β-Hydroxy-5β-androstan-17-one		243°	Prisms	+79°	+314°	78·8	9·0	79·2	8·7	—
3β-Hydroxyandrost-5-en-17-one	$C_{26}H_{32}O_3$	*257°*	Prisms	*+24°*	*+94°*	—	—	—	—	(*b*)
17α-Hydroxyandrost-4-en-3-one		*136–138°*	Prisms	—	—	—	—	—	—	(*d*)
17β-Hydroxyandrost-4-en-3-one		*193°*	Prisms	*+156°*	*+615°*	—	—	—	—	(*b*)
C_{19} steroids: dibenzoates										
5α-Androstane-3β:17β-diol	$C_{33}H_{40}O_4$	198–200°	Needles	+36°	+188°	79·1	7·9	79·2	8·1	—
Androst-5-ene-3β:17α-diol	$C_{33}H_{38}O_4$	*152°*	Needles	*−68°*	*−339°*	—	—	—	—	(*b*)
Androst-5-ene-3β:17β-diol		*217°*	Prisms	*+13°*	*+65°*	—	—	—	—	(*b*)
C_{21} steroids: monobenzoates										
3β-Hydroxy-5α-pregnan-20-one	$C_{28}H_{38}O_3$	*197°*	Prisms	*+76°*	*+319°*	—	—	—	—	(*b*)
3β-Hydroxy-5β-pregnan-20-one		154–155°	Prisms	+80°	+338°	79·3	9·2	79·6	9·0	(*e*)
3β-Hydroxypregn-5-en-20-one	$C_{28}H_{36}O_3$	*197°*	Leaflets	*+40°*	*+168°*	—	—	—	—	(*b*)
3β-Hydroxypregna-5:16-dien-20-one	$C_{28}H_{34}O_3$	171–172°	Needles	−9°	−38°	80·3	7·9	80·3	8·2	(*f*)
3β-Hydroxy-16α-methoxypregn-5-en-20-one	$C_{29}H_{38}O_4$	156–157°	Prisms	−1°	−3°	77·6	8·6	77·3	8·5	(*f*, *g*)
C_{21} steroids: dibenzoates										
5α-Pregnane-3β:20α-diol	$C_{35}H_{44}O_4$	171–172°	Prisms	+27°	+139°	—	—	—	—	(*h*)
5α-Pregnane-3β:20β-diol		237–239°	Needles	−10°	−53°	—	—	—	—	(*h*, *i*)
5β-Pregnane-3α:20α-diol		131–133°	Prisms	+31°	+165°	79·3	8·2	79·5	8·4	(*j*)
5β-Pregnane-3β:20α-diol		169–170°	Prisms	+23°	+123°	—	—	—	—	(*k*)
17α-Methyl-*D*-homoandrostane-3β:17aβ-diol (uranediol)		211–213°	Leaflets	+22°	+118°	—	—	—	—	(*h*)
Pregn-5-ene-3β:20α-diol	$C_{35}H_{42}O_4$	194–196°	Needles	+5°	+26°	80·1	8·2	79·8	8·0	—
Pregn-5-ene-3β:20β-diol		228–230°	Needles	−48°	−252°	80·0	7·8			—
5α-Pregnane-3β:17α:20α-triol	$C_{35}H_{44}O_5$	216–217° 230–231°	Prisms	−3°	−14°	77·5	8·1	77·2	8·1	—
C_{21} steroids: tribenzoates										
5α-Pregnane-3β:16α:20β-triol	$C_{42}H_{46}O_6$	211–213°	Needles	−14°	−91°	—	—	—	—	(*h*)
Uranetriol		234–235°	Needles	+81°	+524°	—	—	—	—	(*h*)

(*a*) Lieberman *et al.* (1948) give $[\alpha]_D$ +3° in ethanol, which must be a misprint. (*b*) Barton & Cox (1948).
(*c*) Lieberman *et al.* (1948) give $[\alpha]_D$ +111° in ethanol, $[M]_D$ +437°. (*d*) Ruzicka & Kägi (1936). (*e*) Recrystallized from methanol.
(*f*) These compounds were prepared from 3β-acetoxypregna-5:16-dien-20-one by hydrolysis with KOH in aqueous methanol, benzoylation and chromatography (cf. Fukushima & Gallagher (1950, 1951), who prepared the corresponding acetates and discovered the nature of the reaction between steroid 16-en-20-ones and alcoholic alkali). (*g*) Found: OCH_3, 7·9, 8·9. $C_{29}H_{38}O_4$ (1 OCH_3) requires 6·9 %.
(*h*) Brooks *et al.* (1952). (*i*) Pearlman (1946). (*j*) Recrystallized from acetone. (*k*) Pearlman (1944).

Table 2. *Chromatography of 20-epimers*

Run	Eluent, light petroleum:benzene (%, v/v)	Fraction no.	Eluate: Weight (mg.)	Eluate: M.p.
Chromatogram I. As benzoates. (15 mg. each of 5α-pregnane-3β:20α-diol dibenzoate and its 20β-epimer. 1·8 g. Al_2O_3; each run 3 ml. eluent.)				
1–7	90:10	I*A*	12·1	236–237°
8 and 9	90:10	I*B*	3·5	156–227°
10	85:15			
11–16	85:15	I*C*	13·0	166–169°
17–23	80:20			
24–26	70:30			
Chromatogram II. As acetates. (15 mg. each of 5α-pregnane-3β:20α-diol diacetate and its 20β-epimer. 1·8 g. Al_2O_3; each run 3 ml. eluent.)				
1–6	90:10	II*A*	7·4	140–142°
7–9	90:10	II*B*	5·5	124–137°
10–12	85:15			
13–16	85:15	II*C*	2·5	132–157°
17–21	80:20	II*D*	3·1	149–159°
22–25	70:30	II*E*	8·5	160–163°
26–28	50:50			

Fraction II*E* recrystallized from methanol gave 4·9 mg. (m.p. 166–168°). The melting points of the pure diacetates are as follows: 3β:20α-diol, 166–168°; 3β:20β-diol, 142–143°.

Table 3. *Chromatography of epimeric and related pairs of hydroxysteroids*

(Mixtures used for elution are all light petroleum:benzene and are described in terms of % benzene. 'Recovery *x*% pure' means that *x*% of each component was separated from the chromatogram in a state of purity such that the m.p. was only a few degrees below the m.p. of an analytically pure sample.)

Types *Epimers at*	Compounds	Result of chromatography: As acetates	Result of chromatography: As benzoates
$C_{(3)}$	3α- and 3β-Hydroxy-5α-androstan-17-ones	Good separation: 3α-epimer eluted by 10–20% benzene; 3β- by 17–100% benzene. Recovery 90% pure	Good separation: 3α-epimer eluted by 20–30% benzene, 3β- by 40–100% benzene. Recovery nearly 100% pure
$C_{(3)}$	3α- and 3β-Hydroxy-5β-androstan-17-ones	—	Good separation: 3α-epimer eluted by 20–40% benzene, 3β- by 40–100% benzene
$C_{(5)}$	3β-Hydroxy-5α- (and 5β-) androstan-17-ones	No apparent separation. Mixtures eluted continuously from 15–100% benzene	Partial separation: mixtures eluted by 25–100% benzene; 5α-compound enriched in early fractions and 5β-compound in later fractions
$C_{(5)}$	3β-Hydroxy-5α- (and 5β-) pregnan-20-ones	—	Partial separation: mixtures eluted by 5–100% benzene; enrichment of 5α-compound in early fractions
$C_{(17)}$	Androst-5-ene-3β:17α- (and 3β:17β-) diols (3-acetates)	Good separation: 17α-epimer eluted by 10–20% benzene, 17β- by 15–100% benzene. Recovery 80% pure	Good separation: 17α-epimer eluted by 15% benzene, 17β- by 20% benzene. Recovery 90% pure
$C_{(17)}$	17α- and 17β-Hydroxyandrost-4-en-3-ones	—	Good separation: 17α-epimer eluted by 35–50% benzene; 17β-epimer eluted by 50–100% benzene. Recovery 85% pure
$C_{(20)}$	Pregn-5-ene-3β:20α- (and 3β:20β-) diols	Partial separation: mixtures eluted by 15–40% benzene; enrichment of 20β-compound in early fractions	Good separation: 20β-epimer eluted by 10% benzene; 20α- by 15–20% benzene. Recovery 90% pure
$C_{(20)}$	5α-Pregnane-3β:20α- (and 3β:20β-) diols	Fair separation. For details see Table 2	Good separation. For details see Table 2
$C_{(20)}$	5β-Pregnane-3β:20α- (and 3β:20β-) diols	—	Good separation: 20β-epimer eluted by 15–20% benzene; 20α-epimer eluted by 20–30% benzene

Table 3 (*cont.*)

Types	Compounds	Result of chromatography: As acetates	Result of chromatography: As benzoates
Other pairs			
5α and Δ^5	3β-Hydroxy-5α-androstan-17-one and 3β-hydroxy-androst-5-en-17-one	Poor separation: mixtures eluted by 20–100% benzene. Slight concentration of unsaturated compound in later fractions*	Poor separation: mixtures eluted by 40–100% benzene. Slight concentration of unsaturated compound in later fractions†
5α and Δ^5	3β-Hydroxy-5α-pregnan-20-one and 3β-hydroxypregn-5-en-20-one	—	Poor separation: mixtures eluted by 7–40% benzene. Slight concentration of unsaturated compound in later fractions‡
Δ^5 and $\Delta^{5:16}$	3β-Hydroxypregn-5-en-20-one and 3β-hydroxypregna-5:16-dien-20-one	—	Poor separation: mixtures eluted by 20–100% benzene. Slight concentration of 5:16-diene compound in late fractions§
17- and 20-Ketones	3β-Hydroxy-5α-androstan-17-one and 3β-hydroxy-5α-pregnan-20-one	Good separation: 20-ketone eluted by 10–30% benzene, 17-ketone by 20–100% benzene. Recovery 90% pure	Good separation: 20-ketone eluted by 15–30% benzene; 17-ketone by 20–100% benzene. Recovery 90% pure

* Fractions eluted by the same solvent mixture were combined and 1 mg. samples were treated with four drops of conc. H_2SO_4. All the fractions developed an immediate yellow-brown colour; after 48 hr. the first fraction was brown and all the others purple-brown. Pure 3β-acetoxyandrost-5-en-17-one gave an immediate orange colour with conc. H_2SO_4 (after 1 hr., orange-brown; 48 hr., violet). 3β-Acetoxy-5α-androstan-17-one under these conditions gave no immediate colour (after 1 hr., orange; 48 hr., reddish brown) (cf. Dirscherl & Zilliken, 1943; Paterson, 1947).

† Each solid fraction was treated with one drop of conc. H_2SO_4; all turned red in a few minutes. After 48 hr. early fractions were brown and later fractions blue. The pure benzoates gave the same H_2SO_4 colour reactions as the corresponding acetates.

‡ Conc. H_2SO_4 (four drops) was added to every fourth fraction. After 5 min. early fractions were yellow and late fractions red. Pure 3β-benzoyloxy-5α-pregnan-20-one gave a pale-yellow colour under these conditions and 3β-benzoyloxypregn-5-en-20-one a dark-orange colour.

§ Selected fractions were dissolved in a mixture of two drops of $CHCl_3$ and three drops of ethanol. On addition of two drops of dinitrophenylhydrazine (1% in ethanol), early fractions gave a light-orange precipitate and late fractions gave a dark-orange precipitate. Pure 3β-benzoyloxypregn-5-en-20-one gave a yellow precipitate in this test while the pure 5:16-diene gave a dark-orange precipitate.

RESULTS

Details of typical chromatograms of 20-epimeric diols as acetates and as benzoates are given in Table 2. The behaviour of the other pairs of compounds examined is described briefly in Table 3.

DISCUSSION

20-Epimeric hydroxysteroids. In all cases, the benzoates separated well, whilst the acetates separated only partially. Previous workers have found that 20-epimeric acetates can be separated only with difficulty by chromatography or crystallization (cf., for example, Reichstein & Gätzi, 1938; Hirschmann & Hirschmann, 1950*a*, *b*; Julian, Meyer, Karpel & Cole, 1951).

The separation of 20-epimers is the only example where chromatography of benzoates gives appreciably better results than chromatography of acetates; but since we have yet found no examples where the use of benzoates is disadvantageous it may be profitable to chromatograph the benzoates first in trying to separate mixtures of natural steroids of unknown composition.

3-Epimeric hydroxysteroids. These can be separated sharply by chromatography, either as acetates or benzoates. Although 3α- and 3β-hydroxysteroids are commonly separated by digitonin, this method is often attended with losses. Furthermore, recent work in this Department (Haslam & Klyne, 1952) extending some observations by Haslewood (1947) has shown that many 3β-hydroxy-5β-steroids are precipitated by digitonin only when present in high concentrations. The separation of such steroids from their 3α-epimers could best be carried out by chromatography of their acetates or benzoates.

Some pairs of 3-epimeric hydroxysteroids have been separated by chromatography as alcohols (Shoppee (1946), 5α-cholestan-3-ols; Ruzicka, Meister & Prelog (1947), 3-methyl-5α-androstan-3-ols; Bridgwater & Shoppee (1952), 5β-cholestan-3-ols (coprostanols)). In each case the 3α-hydroxy epimer was eluted first.

5-Epimeric steroids. 5α- and 5β-Epimeric steroids are difficult to separate by chromatography as alcohols or as acetates (cf. for example, Reichstein & Lardon, 1941; Dobriner, Lieberman & Rhoads, 1948; Wenner & Reichstein, 1944; Lieberman, Dobriner, Hill, Fieser & Rhoads, 1948). The

Table 4. *Conformation of acyloxyl groups and order of elution of epimers from alumina*

(The symbols (*e*) and (*p*) indicate the conformation (equatorial or polar) of the acyloxyl groups concerned.)

Position of epimerism	Type of compound	Order of elution of acetates and benzoates
3	5α-Androstan-3-ols	3α (*p*) before 3β (*e*)*
3	5β-Androstan-3-ols	3α (*e*) before 3β (*p*)*
5	5α- and 5β-Androstan-3β-ols	3β (*e*): 5α before 3β (*p*): 5β
17	Androstan-17-ols	17α (*p*) before 17β (*e*)*

* The same order of elution has been found for the free alcohols (Shoppee, 1946; Bridgwater & Shoppee, 1952; Elks & Shoppee, 1953).

benzoates give results which are a little better, but still disappointing. The chromatographic method used by Dingemanse, Huis in't Veld & de Laat (1946) for the fractionation of urinary 17-ketosteroids permits sharp separation of 5-epimers (3α-hydroxy-5α(and 5β)-androstan-17-ones) as the alcohols, but it could hardly be used for quantities larger than a few milligrams.

5α-Saturated and Δ⁵-unsaturated steroids. The problem of trying to separate related steroids of these types often occurs in dealing with natural steroid mixtures, especially urinary extracts. Lieberman *et al.* (1948) have pointed out the difficulties of this separation (cf. also Seebeck & Reichstein, 1943).

Ruzicka & Prelog (1943) surmounted the difficulty by treating a mixture of saturated and unsaturated compounds (3β-hydroxy-5α-pregnan-20-one and 3β-hydroxy-pregn-5-en-20-one from swine testis) with osmium tetroxide; the unsaturated compound gave a 5α:6α-diol, which could be readily separated from the unchanged saturated steroid, but which could not be reconverted into the Δ⁵-unsaturated compound.

The use of benzoates for chromatography offers little advantage over the use of the acetates.

17-Epimeric hydroxysteroids. These can be separated sharply by chromatography either as acetates or as benzoates. Elks & Shoppee (1953) have found that the epimeric androstan-17-ols can be separated as alcohols, the 17α-epimer being eluted before the 17β-epimer.

17-Ketosteroids and 20-ketosteroids. Related pairs of 3-hydroxy-17(and 20)-ketosteroids can be separated sharply either as acetates or as benzoates. Lieberman *et al.* (1948) showed that such pairs of compounds in urinary extracts could be separated as alcohols, the 20-ketone being eluted first in each case.

Δ¹⁶-20-Ketosteroids and the related 16:17-saturated compounds. One attempt at the separation of a pair of related compounds (3β-hydroxypregn-5-en-20-one and its Δ⁵·¹⁶ analogue) by chromatography of the benzoates gave disappointing results. The conjugated 16-en-20-one was concentrated slightly in the later fractions of the chromatogram.

Conformation of acyloxyl groups and order of elution of epimers. The results of this work were considered in an attempt to correlate the conformation of acyloxyl groups in the steroid skeleton (cf. Barton, 1950; Barton & Rosenfelder, 1951) with the order in which the epimeric compounds were eluted from alumina (as acetates or benzoates). The results, summarized in Table 4, together with a few results from the free alcohols, show no regularity. Dr D. H. R. Barton has suggested to the authors that the lack of regularity here may be due to the fact that when compounds are adsorbed on to a solid surface, they may well take up conformations different from those which are preferred in solution. In contrast, the behaviour of steroid alcohols in partition chromatograms seems to show a consistent relationship to the equatorial or polar nature of the hydroxyl groups. Savard (1953) has shown that on paper partition chromatograms (using propylene glycol as stationary phase and light petroleum (b.p. 60–110°) or other saturated hydrocarbon as moving phase) the alcohols carrying polar hydroxyl groups move faster than their epimers carrying equatorial hydroxyl groups; examples tested include 3-epimers (both in the 5α- and 5β-series) 6-, 7-, 11- and 17-epimers. Presumably the more hindered polar hydroxyl groups in the steroids associate with the hydroxyl groups of the propylene glycol less than do the equatorial hydroxyl groups in the corresponding epimeric steroids.

SUMMARY

1. Different types of epimeric and related hydroxysteroids have been subjected to chromatography as acetates and benzoates.

2. Hydroxysteroids which are epimeric at $C_{(20)}$ can be separated readily by chromatography as benzoates, but not as acetates. Compounds epimeric at $C_{(3)}$ or at $C_{(17)}$ can be separated readily either as acetates or as benzoates.

3. Steroids epimeric at $C_{(5)}$ and some related pairs of steroids (a 5α-saturated compound and the corresponding Δ⁵-unsaturated compound) cannot be separated easily either as acetates or benzoates.

4. Nine new steroid benzoates are described.

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Protein-bound Iodine in Extrathyroidal Tissues

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It has been shown that the concentration of protein-bound iodine in serum usually reflects the concentration of the thyroid hormone, increasing in hyperthyroidism and decreasing in hypothyroidism. Several reasonably satisfactory analytical methods have been developed in the past decade and give results which are in essential agreement. There is evidence that normally most of the protein-bound iodine which is separable from inorganic iodide in serum by zinc precipitation is present as thyroxine (Taurog & Chaikoff, 1948; Man, Kydd & Peters, 1951).

The desirability of determining the concentration of a hormone not merely in the manufacturing gland or in the blood stream, but also in the peripheral tissues, has led to many attempts, beginning with

Justus (1904), to extend the scope of iodine determinations from serum to tissues. Early work consisted of determinations of total, rather than protein-bound iodine, and was based on methods which were unreliable even for analysis of serum. Even the more recent reports give insufficient data regarding the reliability of the methods used. In view of recent progress in analytical methods, a new attempt to solve this problem seemed justified. Little would be gained, however, by adding to the conflicting values already in the literature without giving adequate data on the reliability of the method. We have therefore devoted considerable attention to this point. In this paper we shall describe the method used by us for serum analysis and indicate its limits of error, describe the modifications of this method which make it suitable for tissue analysis, indicate its limits of error when so used, and report the results of tissue analyses.

METHODS

Protein-bound iodine of serum

This is a modification of the method described by Barker (1948) and, like it, follows the general outline of Chaney's (1940) method in that the sample is digested in hot chromic acid and distilled over into a trap containing an alkaline reducing solution, the concentration of I_2 in the distillate being finally determined by its ability to catalyse the reduction of cerium.

Reagents

These are as described by Barker, with the following exceptions:

Double-distilled water. Once-distilled water is redistilled from alkali.

Sodium arsenite solution. 3·75 g. As_2O_3 are dissolved in 100 ml. 20% (w/v) NaOH and made up to 500 ml. This is used in the trap during distillation. No Na_2SO_3 or N-H_2SO_4 are used. This substitution has been found satisfactory by Barker.

Arsenious acid solution. 2 g. NaOH are dissolved in 200 ml. of water and 3·0 g. As_2O_3 and 87·5 g. NaCl are added. To the mixture is then added a solution which has been made by cautiously adding 764 ml. conc. H_2SO_4 (sp.gr. 1·84) to 1 l. of water. The acid solution should be cooled before mixing. After mixing, the solution is again cooled and made up to 2 l.

Standard iodide solutions. Each standard solution is prepared by diluting 40 ml. sodium arsenite solution and the required quantity of a standard solution of KI to 1 l. Thus prepared, 10 ml. of each standard solution contains the same quantity of trap solution as a 10 ml. sample of unknown distillate. These standard solutions are stable indefinitely. We have found that solutions containing 0·00, 0·01, 0·02, 0·06, 0·10 and 0·12 μg. of iodide/10 ml. are sufficient to establish the standard curve.

Haemoglobin solution. Approximately 500 ml. fresh oxalated or defibrinated dog blood are centrifuged and the plasma removed. The erythrocytes are then washed five times by suspension in 0·85% (w/v) NaCl and centrifugation. The volume of NaCl solution used in each wash should be at least equal to the volume of erythrocytes. The erythrocytes are finally added to water and the solution made up to a volume twice that of the original volume of whole blood.

Special apparatus

300 ml. digestion flasks are employed. In the end-point determination an Evelyn colorimeter with a no. 420 filter is used, and the test tubes are the 'selected absorption tubes' designed for this colorimeter. Each stirrer consists of a glass rod, about 25 cm. long, to the lower end of which is sealed a small, slightly concave glass disk, about 1·5 cm. diameter. The disk is sealed at right angles to the stirring rod, with the concavity directed upward, so that the stirrer resembles a shallow spoon with an elongated handle bent to form a right angle with the bowl of the spoon.

Procedure

This follows Barker's method with the following exceptions.

Digestion. The flasks are agitated during initial heating until boiling is smooth and vigorous.

Distillation. Sodium arsenite solution (1 ml.) is placed in the trap at the beginning of distillation. At the conclusion of distillation the trap is drained into a 25 ml. volumetric flask before the flame is turned off, in order to prevent suckback. After the flame is turned off, the still is washed four times with water and the distillate plus washings is made up to 25 ml.

End point. A 10 ml. sample from each 25 ml. of distillate plus washings is placed in a colorimeter tube. Standard iodide solution (10 ml.) is placed in each of twelve test tubes to provide six pairs of standards containing from 0·00 to 0·12 μg. of iodide. Arsenious acid solution (2 ml.) is added to each tube, which is then placed, after thorough shaking, in the previously warmed water bath, the standards being scattered at random among the unknowns. A stirrer is placed in each tube. Ceric ammonium sulphate solution is placed in a separate reservoir tube which is also placed in the bath. After 30 min. equilibration, 0·5 ml. ceric ammonium sulphate solution is withdrawn from the reservoir and added to each tube according to a definite schedule as described by Barker, except that 60 sec. are allowed between tubes. Immediately after addition of ceric ammonium sulphate solution the contents of the tube are vigorously stirred for 15 sec. by repeatedly drawing the stirrer up and down through the solution while the thumb and forefinger rotate the stirrer. A reading is taken 24 min. after addition of the ceric ammonium sulphate solution, the stirrer being removed just before the tube is placed in the colorimeter.

Blanks and duplicates. Each reagent blank contains 2 ml. of haemoglobin solution as 'carrier' protein, instead of the dialysed dog serum recommended by Barker. A pair of blanks is included with each set of unknowns and each unknown is analysed in duplicate. However, after distillation of all blanks and unknowns in a set, only one of each pair of blanks and unknowns is run in the end-point determination. The other member of each pair is set aside and analysed on the subsequent day. Thus, each day's end-point determination includes half of that day's set and half of the previous day's set. The distillates are stable at room temperature for at least 6 days.

Limits of error

The method was tested in three ways: (*a*) amounts of iodide ranging from 0·02 to 0·60 μg. were added to blanks containing haemoglobin solution before digestion, and the recovery and precision determined; (*b*) precision was tested by repeated analyses of the same serum; (*c*) the recovery of 0·1 μg. of iodide added before digestion to a serum of known iodine content was repeatedly determined. The results of these experiments are shown in Tables 1 and 2.

Analyses of sera from euthyroid humans by the present method have shown the normal range of values to be similar to that previously established with the Riggs-Man method (Riggs, 1947).

Protein-bound iodine of tissues

The method just described is used with the following further modifications.

Precipitating reagents

The NaOH solution is not modified, but the concentrations of $ZnSO_4.7H_2O$ and H_2SO_4 in the acid zinc sulphate solution are three times those described by Barker (1948).

Procedure

Sampling of tissues. The dog tissues were taken from carcasses directly after exsanguination under light ether anaesthesia ('bleeder' dogs) or at the conclusion of heart-lung preparations ('HLP' dogs). All tissues were blotted free of gross blood, trimmed, and stored frozen at $-7°$. Only the ventricular muscle of the heart was used. As far as possible, renal cortex was separated from the medulla and only the cortex analysed. Intestine was stripped of the tunica serosa and tunica muscularis, and the contents of the lumen were washed out with tap water. The tunica albuginea was stripped from the testis.

Grinding and precipitation. A 2 g. ($\pm$0·1 g.) sample of the thawed tissue is placed in a mortar with a small amount of alundum (grit no. 36B). As the tissue is ground it is moistened with small amounts of acid zinc sulphate solution. The total amount of this solution used in grinding and precipitation is 16 ml. The thoroughly ground tissue is transferred to a 50 ml. centrifuge tube with the aid of about 10 ml. of water and precipitated by adding the rest of the acid zinc sulphate solution and 6 ml. of 0·75 N-NaOH. Washing is then carried out as described by Barker.

Digestion. 1 l. flasks are used. 75 ml. of 70% (w/v) H_2SO_4 and 9 ml. of 60% (w/v) CrO_3 are used. Haemoglobin solution (6 ml.) is added to each blank. After addition of the reagents and sample the flasks are stoppered and left overnight at room temperature ('cold' digestion) before digestion with heat is carried out.

Distillation. 20 ml. of 50% (w/v) H_3PO_3 are used.

Limits of error

The tissue method has been subjected to the same three types of test as the serum method. The results are listed in Tables 1 and 2. Except when otherwise noted, 2 g. samples of tissue were used, and the results were calculated in terms of wet tissue.

It should be noted that the amounts of iodine added to blanks and tissues in recovery experiments were of the same order of magnitude as those actually found in 2 g. samples of normal dog tissue. In calculating the percentage recovery of the added iodine, the variation between replicate analyses is necessarily exaggerated somewhat since all of the analytical error is ascribed to the added iodine. The standard deviation for the recovery of iodine added to tissue is therefore misleadingly high. The lower the ratio of added iodine to iodine already present, the higher this error will be. If the number of determinations of recovery is relatively small, such an increase in standard deviation may cause serious error in estimating the mean recovery. Table 2 shows that the standard deviation expressed as per cent recovery tended to vary inversely with the amount of added iodine. However, the standard deviation expressed as absolute amount of iodine was fairly constant from tissue to tissue.

Comments and precautions

Storage of tissues. The best conditions for storage of tissues before precipitation have not been precisely determined. However, the protein-bound iodine in a large sample of normal human serum stored at $-7°$ has been determined repeatedly during a period of 88 weeks and has remained constant within the error of the method. A limited number of repeat analyses have indicated that storage of tissues for as long as a month at this temperature is probably also safe.

Grinding and precipitation. Comparison of a series of analyses of dog heart in which 0·7 g. samples were used, with a series in which 2 g. samples were used showed that the latter gave appreciable improvement in precision. In order to produce a precipitate which can be well washed, the tissue must be finely ground before precipitation. The Waring Blendor and the Potter homogenizer gave unsatisfactory results. Grinding by hand with alundum for several minutes reduces even the tougher tissues to a satisfactory pulp. This step increases the total time consumed in the analysis, but prevents falsely high values caused by inefficient washing.

Digestion. In two experiments radioactive iodine (^{131}I) was added to the reagents as used for serum, and all vapours arising during digestion were condensed and collected in fractions. The principal loss of iodine occurred over a 3–4 min. period directly after the onset of boiling in the first digestion, and amounted to only about 2%.

Recoveries were poor when an excess of protein was added and digestion was therefore incomplete. Recoveries were also unreliable when too little protein was added and the resulting excess of CrO_3 was not completely reduced by the H_3PO_3. When no protein was added, but care was taken to reduce all the excess CrO_3 by addition of excess H_3PO_3, recoveries were still unreliable. The necessity for 'carrier' protein has been noted by Taurog & Chaikoff (1946), and by Barker (1948). A solution of dog haemoglobin can be simply prepared and furnishes 'carrier' protein of extremely low iodine content. Human haemoglobin has also been found satisfactory in the thyroid clinic of the Massachusetts General Hospital (Pratt, 1950). If the use of a sample weighing considerably less than 2 g. is unavoidable, enough haemoglobin should be added to bring the total amount of protein to about 400 mg. For tissues it was found necessary to triple the amounts of both digestion reagents since the use of 9 ml. of CrO_3 with 25 ml. of H_2SO_4 led to very poor recoveries.

Table 1. *Precision of protein-bound iodine method for human serum and dog tissues*

Material analysed	Dog	Preliminary 'cold' digestion	No. of pairs* of analyses	Protein-bound iodine: Mean ± S.D. (μg./100 g.)	Protein-bound iodine: Coefficient of variation 100 S.D./M
Normal human serum	—	No	11	6·1 ± 0·19	3·1
Heart	*D*	No	3	1·7 ± 0·07	4
Heart	*E*	No	3	2·2 ± 0·31	14
Skeletal muscle	*K*	No	6	1·2 ± 0·37	31
Skeletal muscle	*M*	Yes	5	1·1 ± 0·31	28
Liver	*J*	No	6	6·1 ± 0·68	11
Liver	*K*	Yes	5	5·4 ± 0·98	18
Spleen	*K*	No	6	1·9 ± 0·24	13

* As explained in the text, one member of any given pair was usually digested and distilled on a different day from its mate and the two were never run in the same end-point determination. Furthermore, in all experiments listed in Tables 1 and 2, except those involving heart, the individual analyses contributing to any statistic were spread over four or more end-point determinations.

Table 2. *Recovery of iodine added to reagents, serum and tissues*

(2 g. samples of tissue used except where otherwise stated.)

Dog*	Material to which iodine was added	Iodine added (μg.)	Form in which iodine added and time of addition†	No. of pairs of analyses	Percentage recovery. Mean ± S.D.
	Blank + haemoglobin (no precipitation)	0·02	KI, before digestion	8	97 ± 19
		0·10		20	98 ± 4·8
		0·60		6	87 ± 1·2
	Normal dog serum (no precipitation)	0·10	KI, before digestion	8	92 ± 2·8
	Blank + haemoglobin (no precipitation)	0·02	KI, before digestion	8	87 ± 9·6
		0·04		12	85 ± 7·4
		0·06		10	88 ± 7·9
		0·10		13	105 ± 7·2
		0·60		6	89 ± 1·1
D	Heart	0·06	KI, after pptn. and washing, before digestion	3	97 ± 3·2
E	Heart, 1·5–2·0 g.	0·061	1 ml. of normal human serum, before grinding	4	91 ± 12
K	Skeletal muscle	0·05	KI, after pptn. and washing, before digestion	8	36 ± 29
M	Skeletal muscle	0·05	KI, after cold digestion before hot digestion	6	95 ± 3·7
M	Skeletal muscle	0·05	KI, after pptn. and washing, before cold digestion	5	81 ± 13
J	Liver	0·08	KI, after pptn. and washing, before digestion	8	84 ± 4·6
K	Liver, 1·3 g.	0·134	2 ml. normal human serum after pptn. and washing, before cold digestion	6	81 ± 14
K	Spleen	0·04	KI, after pptn. and washing, before digestion	8	74 ± 19

* See Table 1 for the iodine content of the dog tissues used in these experiments.
† When 'digestion' alone is listed, a preliminary cold digestion was not used.

A preliminary 'cold' digestion was adopted after it was found that iodide added to certain tissues, especially skeletal muscle, could not be recovered reliably unless a preliminary 'cold' digestion was done. A comparison of the data for dog *K* skeletal muscle with those for dog *M* in Table 2 will make this clear. Although the recovery of the protein-bound iodine already present in the tissue may have been adequate (Table 1), even when the recovery of added iodide was poor, such an assumption was considered dangerous. We cannot explain why the recoveries of iodide added to certain other tissues were satisfactory in the absence of preliminary digestion (Table 2). It was decided that the safest course would be to subject all tissues to a preliminary 'cold' digestion. It seemed very unlikely that this would be detrimental with tissues which gave good recoveries without such digestion. This assumption was tested by comparing the recoveries from liver with and without 'cold' digestion. No significant difference was noted (Table 2).

Distillation. The size of the flasks has been increased to accommodate the increased volume of reagents. The small flasks used for serum analysis can be used for tissue analysis only if the H_3PO_3 is added very slowly, thus preventing the digest from boiling up into the trap.

End-point determination. The schedule used is arbitrary and may be modified as desired, provided each tube is treated in exactly the same way.

Since the reaction proceeds most rapidly directly after addition of the cerium, immediate thorough mixing is necessary. Shaking the tubes in the bath or stirring with an ordinary stirring rod gave poor results. Removing the tubes from the bath and shaking allowed satisfactory mixing but caused appreciable cooling. Satisfactory mixing without removal of the tubes was obtained with the stirrers described above. They are easy to make and to clean.

Originally, the end-point determination for both members of each pair of duplicates was performed at the same time. However, experiments in which a large number of analyses of the same serum or tissue were performed showed that the variation between results obtained on the same day was significantly less than the variation between results obtained on different days. The principal source of this day-to-day variation was the end-point determination, as the following experiment showed. On day 1, 0·10 μg. of iodide was added to each of six blanks and the recoveries were determined. On day 2, a second set of six was similarly analysed. Colorimetric analysis of each distillate of the first set was also performed on day 2 together with the distillates of the second set. A significant difference ($P = 0{\cdot}01$) was found between the means of the results for the first set of distillates analysed on day 1 and day 2 in two different end-point determinations. No significant difference was found between the means of the results of the two different sets of analyses, digested and distilled on different days, when the same end-point determination was used for both sets. The same batch of reagents was used in both end-point determinations, and a standard curve was run for each determination. This experiment led us to adopt the system of splitting duplicates between two end-point determinations, with consequent decrease in error due to the differences between end-point determinations.

During any single end-point determination the temperature of the water bath has remained constant within $\pm 0{\cdot}5°$. This amount of variation does not affect the precision of the method appreciably, although we do not deny the desirability of regulation to within $\pm 0{\cdot}1°$, as recommended by Barker (1948). The setting of our thermoregulator has varied spontaneously from day to day between extremes of 35 and 38°. However, this day-to-day variation is of little consequence since a separate standard curve is constructed for each end-point determination, and we have not been able to correlate the differences between the results obtained in different end-point determinations with the observed differences in temperature.

Throughout this paper the mean of each pair of analyses has been treated as a single result. In practice, whenever our results show a difference of more than 2·0 μg./100 g. between the members of a pair of analyses of serum or tissue, the analyses are repeated. In calculating the statistics listed in Tables 1 and 2, however, such data have not been discarded, and the statistics in certain instances therefore slightly overestimate the variation obtained in practice.

The reasons for changes in the arsenious acid solution have been explained in a previous paper (Carr, Graham, Ober & Riggs, 1950). Since then the concentration of NaCl has been further increased. With lower NaCl concentrations the sensitivity of an occasional end-point determination was very low. Increasing the NaCl concentration has eliminated this difficulty. It is conceivable that such poor results were due to contamination with traces of Ag, which is known to interfere with the iodide catalysis, presumably through formation of insoluble AgI (Sandell & Kolthoff, 1936). The solubility product of AgCl is almost 10^6 times that of AgI and it would therefore require a high Cl/I ratio to favour AgCl formation. It is even possible that the entire chloride effect is due to the presence of traces of Ag.

When the amount of iodine in the sample is high, it may be necessary to use less than 10/25 of the distillate. Although the standard solutions contain the same amount of trap solution as 10/25 of a distillate, they may still be used, since the differences in amount of trap solution have not been found important.

In another experiment equal samples of the same pooled distillate from tissue were placed in each of a series of tubes, amounts of iodide ranging from 0·00 to 0·08 μg. were added, and an end-point determination performed. By comparison with the usual series of standard solutions, it was found that the distillate decreased the slope of the standard curve. Distillate from liver (9 ml.) gave results which were 19% too low and 8 ml. of distillate from muscle gave results which were 16% too low. The amount of trap solution was the same in all tubes. Failure to obtain 100% recovery of iodine added to tissue therefore does not necessarily mean loss during digestion or distillation.

RESULTS

The results of analyses of serum and tissues from normal and hyperthyroid dogs are shown in Tables 3 and 4. All dogs were fed a diet of chopped horse meat. HLP (heart-lung preparation) dogs were made hyperthyroid by the additional daily feeding of 0·8 g./kg. of U.S.P. thyroid for 5–8 weeks. Hyperthyroid HLP dogs showed weight loss or increased appetite or both. They also showed a marked tendency to develop hyperthermia during sodium pentobarbital anaesthesia, which was induced once a week for the performance of other

studies (Riggs, Stanbury & Carr, 1952). 'Bleeder' dogs were made hyperthyroid in the same manner, except that the thyroid powder was administered for 1–2 weeks. As a control, one dog was fed 1·6 mg. of inorganic iodide daily for 17 days. This amount was equivalent to the total iodine content of 0·8 g./kg. of the thyroid powder.

The feeding of powdered thyroid clearly increased the protein-bound iodine in the serum and tissues. The protein-bound iodine in the serum and tissues of the dog fed supplementary iodide also increased. To find whether the high concentrations in the latter dog actually represented protein-bound iodine or simply iodide which had not been removed by washing, the iodide in the supernatant fluid from the final wash of each sample of tissue was determined. In the supernatants from skeletal muscle, a mean of 0·052 μg. of iodide was found. If one makes the conservative estimate that one-half this quantity remained in the precipitate, approximately 38 % of the supposed protein-bound iodine may actually have been iodide. For heart, the corresponding figure was 16 %. However, for all of the other tissues less than 10 % of the total iodine found in the precipitate could have represented residual iodide.

Analyses of brain have not been satisfactory, although our results suggest that the concentration of protein-bound iodine in dog brain is very low. Attempts were also made to determine the concentration of protein-bound iodine in the adrenal gland and ovary, but because of the small quantity of tissue available the results were not trustworthy and have been omitted.

In addition to comparing the concentrations of protein-bound iodine in different tissues, we studied the distribution of protein-bound iodine within the cell. These experiments were carried on in collaboration with Dr F. A. Lipmann and Dr R. K. Crane of the Massachusetts General Hospital, who have very kindly given us permission to include the results in this paper. In each of two separate experiments the livers of three normal adult rats were pooled, ground in a homogenizer in sucrose solution, and subjected to differential centrifugation according to the technique described by Hogeboom, Schneider & Pallade (1948) with minor modifications (Lipmann, 1951). Portions of the original suspension and of each fraction obtained by centrifugation were then analysed according to the procedure for tissue except that no grinding was necessary. The results are shown in Table 5. The concentrations of protein-bound iodine appeared to be about equal in the fractions of rat-liver cells. The total amount of protein-bound iodine, calculated from the sum of the three separate fractions, was 79 % of the amount actually found by analysis of the whole suspension in Exp. 1, and 90 % in Exp. 2. Assuming

Table 3. *Protein-bound iodine in serum and tissues of normal dogs*

(μg./100 g. wet wt. tissue)

Dog	Serum	Heart	Skeletal muscle	Liver	Kidney	Spleen	Small intestine	Pancreas	Testis	Lung
				Adult bleeder dogs (male)						
Q	1·8	1·1	0·3	4·6	2·8	0·8	2·3	1·2	1·0	2·3
R	1·5	1·6	0·6	6·5	2·3	1·7	0·5	0·9	0·5	2·4
U	1·1	0·6	0·8	6·4	3·2	3·0	2·4	1·3	1·5	3·7
Mean	**1·5**	**1·1**	**0·6**	**5·8**	**2·8**	**1·8**	**1·7**	**1·1**	**1·0**	**2·8**
S.E.	±0·20	±0·29	±0·15	±0·62	±0·48	±0·64	±0·62	±0·12	±0·29	±0·45
P for serum v. tissue	—	—	—	—	—	—	>0·1	>0·1	>0·1	>0·05
				Adult heart-lung preparation dogs (female)						
B	1·8	0·3	0·3	4·4	3·5	3·7				
G	1·7	1·5	1·4	7·8	1·8	5·0				
Mean, all adult dogs	**1·6**	**1·0**	**0·7**	**5·9**	**2·5**	**2·8**				
S.E.	±0·13	±0·25	±0·20	±0·64	±0·30	±0·74				
P for serum v. tissue	—	<0·1	—	<0·05	<0·05	—				
		>0·05	<0·05	<0·05	<0·05	>0·1				
				12-week puppies (female)						
D	2·6	2·6	1·6	6·9	—					
E	3·0	2·5	2·3	3·3	—					

Table 4. *Protein-bound iodine in serum and tissues of adult dogs fed thyroid or KI*

(μg./100 g. wet wt. tissue)

Drug	Weeks	Dog*	Serum	Heart†	Skeletal muscle	Liver	Kidney	Spleen	Small intestine	Pancreas	Testis	Lung
Thyroid	2	*N*	16·5	(48·5)	6·5	21·5	33·5	21·6	32·4‡	9·2	9·9	27·1
	2	*O*	9·3	7·9	4·3	20·5	20·6	49·3	96·2	7·5	7·6	33·3
	1·5	*P*	10·6	13·3	3·6	10·8	12·2	8·0	17·5	4·3	3·4	14·2
	2·5	*S*	12·5	—	11·9	—	—	—	—	—	—	—
Mean, dogs *N, O, P, S*			**12·2**	**10·6**	**6·6**	**17·6**	**22·1**	**26·3**	**48·7**	**7·0**	**7·0**	**24·9**
Thyroid	5	11	7·8	8·4	(42·6)	—	—	—	—	—	—	—
	6	14	29·9	7·7	—	47·2	—	—	—	—	—	—
	6	15	10·5§	5·1	14·7	32·3	—	—	—	—	—	—
	6	16	15·0§	7·7	9·8	26·4	—	—	—	—	—	—
	5	19	17·9	4·3	7·3	20·5	15·1	—	—	—	—	—
	6	20	8·8	4·9	13·7	25·9§	9·7	6·1	—	—	—	—
	6	21	14·8§	12·8	3·1	24·9	14·4	17·2	—	—	—	—
	8	23	13·8	1·3	6·8	19·3	—	—	—	—	—	—
	5	24	21·8	18·2	7·5	17·7	—	45·7	—	—	—	—
	6	25	9·9	5·8	3·0	11·6	—	—	—	—	—	—
	5	26	12·3	6·3	5·1	—	—	—	—	—	—	—
Mean all dogs fed thyroid			**14·1**	**8·0**	**7·5**	**23·2**	**17·6**	**24·6**	**48·7**	**7·0**	**7·0**	**24·9**
KI	2·5	*T*	5·6	6·8	3·4	15·8	10·8	15·8	46·3	6·8	2·9	26·1
Mean of normal dogs *O, R, U* (Table 3)			**1·5**	**1·1**	**0·6**	**5·8**	**2·8**	**1·8**	**1·7**	**1·1**	**1·0**	**2·8**

* Dogs *N*, *O*, *P* and *S* and *T* were 'bleeder' dogs. All others were HLP dogs.

† Additional blood required in the heart-lung preparation was taken from hyperthyroid dogs for dogs 21–26, and from normal dogs for dogs 11–20. Only the heart was exposed to the added blood.

‡ Mean of the results of two pairs of samples, the means of the pairs agreeing within 2·0 μg. %.

§ The difference between duplicates was greater than 2·0 but less than 4·0 μg. %.

The two values in parentheses have been excluded in calculating the means.

Table 5. *Protein-bound iodine in rat-liver fractions*

(In each of two experiments the livers of three normal adult rats were pooled, dispersed in sucrose solution and differentially centrifuged.)

Fractions		Total protein (g.)	Protein-bound iodine (μg./g. of protein)	Total protein-bound iodine (μg.)
Nuclei	1	0·488	0·30	0·15
	2	0·426	0·26	0·11
Mitochondria	1	2·400	0·31	0·74
	2	2·192	0·38	0·83
Supernatant*	1	2·048	0·23	0·47
	2	1·982	0·35	0·69
Whole suspension (calculated)	1	4·936	—	1·36
	2	4·600	—	1·63
Whole suspension (found)	1	5·448	0·32	1·74
	2	5·320	0·34	1·81

* Chiefly cytoplasm and extracellular fluid.

that roughly 20% of the wet weight of liver is protein, the 0·32 and 0·34 μg. of protein-bound iodine per g. protein found in the suspensions would correspond to protein-bound iodine concentrations of 6·4 and 6·8 μg./100 g. in whole liver. These calculated concentrations are reasonable, since 4·8 and 6·9 μg./100 g. of protein-bound iodine were actually found by analysis of whole, untreated livers from two rats in the same colony.

DISCUSSION

Protein-bound iodine was found in all tissues analysed, but not in equal concentrations. In normal dogs (Table 3) the concentration of protein-bound iodine in skeletal muscle was significantly lower, and in liver and kidney significantly higher than in serum. Indeed the concentration in liver was significantly greater than in any of the other tissues. Albert, Rall, Keating, Power & Williams (1949), Albert & Keating (1949) have reported accumulation of thyroxine in the human liver as judged by *in vivo* measurements of radioactivity, after the intravenous administration of radioactive DL-thyroxine. Although in our experiments the concentration of protein-bound iodine in heart tended to be lower, and in lung higher than in serum, the differences were not significant by Student's '*t*' test. Of all organs studied, the spleen showed the greatest variability. This was to be expected since erythrocytes lack protein-bound iodine (Riggs, Lavietes & Man, 1942) and the quantity of erythrocytes may differ greatly from spleen to spleen.

In discussing the fate of radioactive DL-thyroxine in a cretin, Albert & Keating (1949) noted that on intravenous administration the initial volume of distribution of labelled thyroxine was approximately equal to the plasma volume. After 6 hr., however, a second volume of distribution was attained. 'Perhaps fortuitously this second volume of distribution has approximately the dimensions of extracellular fluid, and is smaller than the volume of distribution of inorganic iodide' (Albert & Keating, 1949). Rough calculations based on the data for three dogs suggest that in these animals the volume of distribution of protein-bound iodine was substantially greater than the volume of extracellular fluid. Furthermore, the concentration of protein-bound iodine per gramme of wet tissue was greater in many tissues than in serum. Such high concentrations indicate that most of the protein-bound iodine in these tissues was extravascular and very probably that it was intracellular, since it is unlikely that a substance so extensively bound to protein would be more concentrated in interstitial fluid than in serum. Finally, the studies of distribution in rat liver (Table 5) strongly suggest that at least some protein-bound iodine is located within cells.

The feeding of U.S.P. thyroid in a dose of 0·8 g./kg. of body weight per day increased the concentrations of protein-bound iodine in serum and in most tissues roughly ninefold (Table 4). The average increase was somewhat less for liver, possibly because of its ability to excrete large quantities of exogenous thyroxine when given in unphysiological doses (Gross & Leblond, 1947). The average increase was somewhat greater for small intestine, very likely in part because of the oral route of administration and perhaps in part because of excretion of organic iodine in the bile. Unfortunately, the significance of the protein-bound iodine found in the serum and tissues of the hyperthyroid dogs is impossible to evaluate since very similar, although in general somewhat lower, concentrations were found in the tissues of the dog given only iodide in an amount equal to the total iodine content of the thyroid used. The only conclusion which may fairly be drawn is that in no tissue was there any striking differential accumulation of the exogenous hor-

mone. An increase in serum protein-bound iodine in humans due to the administration of iodide has been described by Danowski, Johnston & Greenman (1950). By extracting with butanol, Man *et al.* (1951) have been able to separate the protein-bound iodine fraction, which presumably represents hormone from the unknown protein-bound iodine compound and which appears during the administration of inorganic iodine. In future studies of the distribution of exogenous hormone in tissues it would probably be advisable to employ butanol extraction as an integral part of the method.

SUMMARY

1. A method for the determination of protein-bound iodine in extrathyroidal tissues is described. Data regarding the limits of error of the method are given.

2. In normal dogs the concentration of protein-bound iodine in the serum is higher than in skeletal muscle and lower than in liver and kidney. No significant difference was found between the concentration of protein-bound iodine in the serum and that in the heart, lung, spleen, small intestine, pancreas and testis.

3. The feeding of powdered thyroid or iodide to normal dogs caused an increase in the concentration of protein-bound iodine of all tissues studied.

4. Protein-bound iodine is probably partly intracellular in the dog. In rat liver, protein-bound iodine occurs in approximately equal concentrations in the nuclear, mitochondrial, and supernatant (cytoplasm plus extracellular fluid) fractions.

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Studies in Detoxication

48. URINARY METABOLITES OF 4:6-DINITRO-*O*-CRESOL IN THE RABBIT

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4:6-Dinitro-*o*-cresol (DNOC) has been used as an insecticide and fungicide for many years, and more recently has become important in agriculture as a selective weed killer (Shepard, 1951). Its use for this purpose has been the cause of several deaths and its former use in the treatment of obesity has been discontinued because of its high toxicity (Bidstrup & Payne, 1951; Horner, 1942).

Little is known about the metabolism of DNOC; only a small amount is eliminated unchanged in the urine, although it is readily absorbed (Spencer, Rowe, Adams & Irish, 1948). In man, it seems to act as a cumulative poison and is eliminated slowly over a period of weeks after ingestion (Harvey, Bidstrup & Bonnell, 1951; King & Harvey, 1952). In laboratory animals, however, there appears to be no

evidence of accumulation, although the DNOC eliminated in the urine is still only a small proportion of the dose (Parker, Barnes & Denz, 1951).

The toxic action of DNOC can probably be attributed to its blocking of 'oxidative phosphorylation' (Judah, 1952) and it is likely that two of its possible metabolites, 6-amino-4-nitro-*o*-cresol and 4-amino-6-nitro-*o*-cresol have no such inhibitory properties since the two corresponding metabolites of 2:4-dinitrophenol have no such action (Judah & Williams-Ashman, 1951). The object of the present work was to find out whether or not these compounds were metabolites of DNOC and to see if the formation of such metabolites could account for the reported low elimination of DNOC in the urine.

MATERIALS AND METHODS

Reference compounds. 4:6-Dinitro-*o*-cresol, DNOC (Pest Control Ltd.), had m.p. 86° after recrystallization from 95% ethanol and was chromatographically homogeneous. 6-Amino-4-nitro-*o*-cresol, m.p. 178°, was prepared according to Cazeneuve (1897). 6-Acetamido-4-nitro-*o*-cresol had m.p. 209° (see Friedländer, 1927–9). 4-Acetamido-6-nitro-*o*-cresol had m.p. 219° and on deacetylation gave 4-amino-6-nitro-*o*-cresol, m.p. 122° (Nietzki & Ruppert, 1890).

Since DNOC could undergo oxidation of its methyl group *in vivo*, the following derivatives of salicylic acid were prepared; 3:5-dinitrosalicylic acid, m.p. 172°, 3-amino-5-nitrosalicylic acid, m.p. 229°, and 3-acetamido-5-nitrosalicylic acid, m.p. 246° (Meldola, Foster & Brightman, 1917).

Chromatography. Downward paper chromatograms were run in the usual way at room temperature in rectangular glass tanks closed at the top with plate-glass sheet. No special precautions were taken to make the tanks completely airtight or to equilibrate paper with the solvent. Under these conditions the R_F was not exactly the same in different tanks but the general pattern and order of separation were reproducible. All identification of compounds by their R_F values were by comparison with an authentic sample run on the same paper at the same time.

For most purposes Whatman no. 1 or 4 paper was used except where larger amounts were handled, when the thicker no. 3 was found more suitable.

Solvents were prepared by shaking the components together in the proportions by volume shown in Table 1 and the top layer used for chromatography. Values of R_F obtained with the different solvents are quoted in this table to illustrate the degree of separation achieved.

Colour reactions. The following reagents were used to locate compounds on the paper chromatograms (see also Table 2): 2 N-NaOH gave yellow, pink or orange colours with nitro compounds. Ammoniacal $AgNO_3$, prepared from 10 ml. 0·1 N-$AgNO_3$ and 1 drop 40% NaOH with enough dilute ammonia to give a clear solution, gave brown or black spots with aminophenols or di-amino compounds on standing at room temperature for a few minutes. Diazo reagents: a spray of 1% $NaNO_2$ in dilute acetic acid followed a few minutes later by a spray of 1% β-naphthol in N-NaOH,

Table 1. *Paper chromatography of DNOC and its possible metabolites. Relative R_F values with Whatman no. 1 paper**

	Benzene/acetic acid/water 1 : 1 : 2 R_F	*n*-Butanol/acetic acid/water 4 : 1 : 5 R_F	Benzene/*n*-butanol/ammonia (sp.gr. 0·88) 2 : 5 : 2 R_F
4:6-Dinitro-*o*-cresol	0·96	0·95	0·50
6-Acetamido-4-nitro-*o*-cresol	0·84	0·95	0·44
4-Acetamido-6-nitro-*o*-cresol	0·80	0·95	0·38
6-Amino-4-nitro-*o*-cresol	0·56	0·95	0·20
4-Amino-6-nitro-*o*-cresol	0·88	0·95	0·22
3-Amino-5-nitrosalicylic acid	0	0·64	0·13
3-Acetamido-5-nitrosalicylic acid	0	0·56	0·07
6-Acetamido-4-nitro-*o*-cresyl glucuronide	0	0·68	0·07

* Chromatograms run until front had moved 18 in. from the starting point.

Table 2. *Colour reactions of DNOC and related compounds*

Compound	Colour given in aqueous solution with: Aqueous $FeCl_3$	N-NaOH	Diazo test*
DNOC	N.C.	Yellow	—
6-Amino-4-nitro-*o*-cresol	Solution darkens	Red	Yellow with HNO_2, no coupling
6-Acetamido-4-nitro-*o*-cresol	N.C.	Orange-red	Yellow with HNO_2, no coupling
4-Amino-6-nitro-*o*-cresol	N.C.	Pink	Red-violet slowly
4-Acetamido-6-nitro-*o*-cresol	N.C.	Orange-yellow	N.C.
3:5-Dinitrosalicylic acid	Red	Yellow	N.C.
3-Amino-5-nitrosalicylic acid	Red-brown	Orange	Yellow with HNO_2, violet on coupling
3-Acetamido-5-nitrosalicylic acid	Red-brown	Yellow	N.C.

* Diazo test is carried out by acidifying the solution with dilute HCl, adding a drop of 0·1% $NaNO_2$, followed by 1% ammonium sulphamate, then coupling with 1% ethanolic dimethyl α-naphthylamine.
N.C. means no change on adding reagent.

gave red or red-violet colours with amino compounds, but with compounds containing the *o*-aminophenol structure, a yellow or brown colour appeared with the first spray. 10% NaCN: on spraying with NaCN and then heating to 100° for 10 min., DNOC and *m*-dinitro compounds gave pink colours.

Spectroscopy. Wherever amounts of material were sufficient, the spots were examined in the Unicam SP 500 spectrophotometer after elution by 0·1 N-NaOH or ethanol as previously described (Robinson, Smith & Williams, 1951). In many cases, however, the amount of material available on the chromatogram was very small and difficulty was experienced with a considerable irrelevant absorption at wavelengths below about 350–400 mμ. This was not appreciably reduced by washing the paper, either in the chromatographic solvents or ethanol. As far as possible the effect of irrelevant absorption was minimized by eluting, with the same volume of eluent, a strip cut from the paper alongside the spot under investigation and using this in the compensating cell in the spectrophotometer. Absorbing material from the paper, however, may account for only part of the irrelevant ultraviolet absorption, for the extracts of rabbit urine contain much material not connected with DNOC which may underlie the yellow spots eluted. In general, using the methods of extraction described below, amounts of the order of 200 μg. were found necessary to give satisfactory spectra after elution, since the eluate could then be diluted to reduce the relative amount of interfering absorption.

Wherever the spectra had characteristic bands at a wavelength not masked by the irrelevant absorption, identification of the eluted compounds was made by comparison with spectra of authentic material in 0·1 N-NaOH and 0·1 N-HCl.

Spectra of reference compounds and those of metabolites are summarized in Table 3 and typical identifications are shown in Figs. 1–3.

Animal experiments

Animals and dosing. All compounds were administered orally with water by stomach tube. In all, some forty chinchilla rabbits were given 20–30 mg./kg. of DNOC and survived. Two rabbits which had received respectively 31 and 32 mg./kg. of DNOC died within 24 hr. of dosing. Heymans & Casier (1935) have reported that rabbits tolerated subcutaneous injections of 10–20 mg./kg.

6-Amino-4-nitro-*o*-cresol and its N-acetyl derivative were administered in the same way but larger doses were tolerated, no deaths occurring after doses of 600 mg./kg. while some deaths occurred at a dose level of 700 mg./kg. of the amino compound.

Examination of DNOC urine. The urine was normal in appearance and did not react with Benedict solution or ammoniacal $AgNO_3$. The naphthoresorcinol reaction was not different from that of normal urine. In the diazo reaction a slight increase of yellow colour was noted on addition of HNO_2 and on coupling with dimethyl-α-naphthylamine a weak red colour developed. This diazo reaction was still positive 2 days after feeding and was detectable on the third and fourth days.

Since most of the material appeared to be present in the urine of the first 2 days, this was often pooled and worked up together.

Three extracts of the urine were made using peroxide-free ether in a continuous extractor.

Table 3. *Absorption spectra of authentic DNOC and related compounds and those of various fractions from the urine of rabbits dosed with DNOC*

	Absorption spectra					
	In 0·1 N-HCl		In 0·1 N-NaOH			
Compound or extract	$\lambda_{max.}$ (mμ.)	$\epsilon_{max.}$ or $E_{max.}$	$\lambda_{max.}$ (mμ.)	$\epsilon_{max.}$ or $E_{max.}$	$\lambda_{max.}$ (mμ.)	$\epsilon_{max.}$ or $E_{max.}$
DNOC						
Authentic	268	12 600	265	5 000	370 ~ 408–410	12 800 approx. 10 000
From extract *A*	—	—	265	0·36	370 ~ 408–410	0·61 0·48
From extract *B*	—	—	—	—	370	—
6-Acetamido-4-nitro-*o*-cresol						
Authentic	280	6 400	285	6 700	425	13 900
From extract *A*	—	—	285	0·35	425	0·39
6-Amino-4-nitro-*o*-cresol						
Authentic	225 310	8 600 8 500	272	6 100	460	14 600
From extract *B*	—	—	272	0·47	460	0·56
From extract *C*	225 310	0·37 0·35	272	0·31	460	0·64
3-Amino-5-nitrosalicylic acid						
Authentic	—	—	292·5	5 750	457·5	16 000
From extract *B*	—	—	~ 290	—	455	—

~ Denotes an inflexion.

Extract *A* was prepared from the urine which had been made acid (pH 2–5). The residual urine was then made 2N with H_2SO_4, boiled for 3 hr., cooled and then extracted to give extract *B*. The hydrolysate was then neutralized to pH 7–8 and extracted again to give extract *C*. Each extract was reduced to a small volume on the water bath, taken up in ethanol and small portions used for the paper chromatograms.

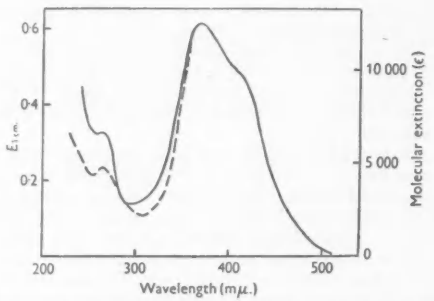


Fig. 1. Absorption spectra of authentic DNOC in 0·1N-NaOH (broken line; ordinate scale, ε, on the right) and of DNOC obtained by paper chromatography of extract *A* of DNOC urine (full line; ordinate scale, $E_{1\,cm.}$, on left).

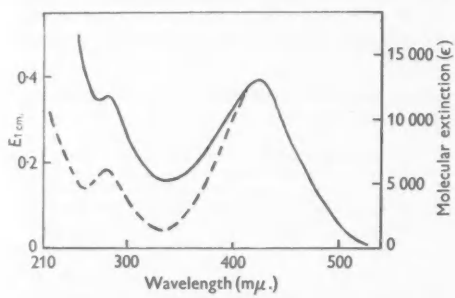


Fig. 2. Absorption spectra of authentic 6-acetamido-4-nitro-*o*-cresol in 0·1N-NaOH (broken line; ordinate scale, ε, to the right) and of the same compound obtained by paper chromatography of extract *A* of DNOC urine (full line; ordinate scale $E_{1\,cm.}$, to the left).

Extract A. This was found to contain unchanged DNOC and metabolites with an unconjugated OH group.

Unchanged DNOC was separated by chromatography in benzene/*n*-butanol/ammonia and identified by comparison with a reference spot of the authentic compound in this solvent and in *n*-butanol/acetic acid/water and benzene/*n*-butanol/ammonia, by its reaction with NaCN, by its yellow colour with 2N-NaOH, and by its absorption spectra after elution with ethanol (cf. Fig. 1). By calculation from the extinction of the absorption band in 0·1N-NaOH at 370 mμ. it was found, in two experiments, that 3·5 and 4% of the dose was eliminated as unchanged DNOC on the first day after feeding. A further 1·5% of the dose was excreted unchanged on the second day. Known amounts of DNOC applied to paper chromatograms and estimated in this way could be recovered quantitatively.

6-Acetamido-4-nitro-*o*-cresol was identified by comparing it chromatographically with an authentic sample in benzene/acetic acid/water, *n*-butanol/acetic acid/water and benzene/*n*-butanol/ammonia, by its yellow colour with 2N-NaOH and by its absorption spectrum after elution from the paper in 0·1N-NaOH (cf. Fig. 2). Its identity was further checked by hydrolysing a portion of the eluate by boiling in 2N-HCl for 2 hr. when the spectrum in 0·1N-NaOH corresponded with that of 6-amino-4-nitro-*o*-cresol.

Calculations from the extinctions in alkaline solution at 425 mμ. showed that about 1·5% of the dose of DNOC was eliminated as unconjugated 6-acetamido-4-nitro-*o*-cresol on the first 2 days after dosing.

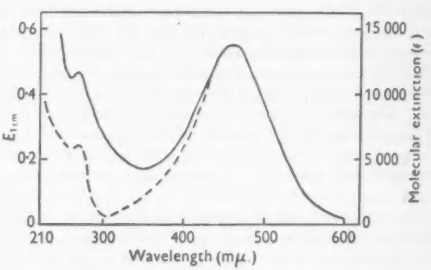


Fig. 3. Absorption spectra of authentic 6-amino-4-nitro-*o*-cresol in 0·1N-NaOH (broken line; ordinate scale, ε, to the right) and of the same compound obtained by paper chromatography of extract *C* of DNOC urine (full line; ordinate scale, $E_{1\,cm.}$, to the left).

6-Amino-4-nitro-*o*-cresol also appeared to be present in small amounts in extract *A* since a spot with the same R_F value as the authentic material in benzene/acetic acid/water and in benzene/*n*-butanol/ammonia was obtained. This spot gave an orange colour with 2N-NaOH and a brown colour with HNO_2. Insufficient amounts were present for spectroscopic identification.

Extract B. This contained compounds liberated from conjugates by hydrolysis and extractable from acid solution.

DNOC was identified, as in extract *A*, by colour reactions, R_F values and spectroscopy, and accounted for 0·7% of the dose as estimated by the extinction in alkaline solution at 370 mμ.

3-Amino-5-nitrosalicylic acid also appeared to be present in some experiments, since a spot was obtained with the same R_F value as the authentic material, in benzene/*n*-butanol/ammonia, *n*-butanol/acetic acid/water and benzene/acetic acid/water and which gave the same red colour with 2N-NaOH. Two-dimensional chromatograms in the second and third of these solvents led to a clear separation of this compound from 6-amino-4-nitro-*o*-cresol which gives similar colour reactions. Elution of the spot in methanol and examination of its spectrum in 0·1N-NaOH showed that

it had a maximum at 450 mμ. and an inflexion at 295 mμ. The authentic compound had a maximum at 457·5 and 292·5 mμ., but the amount of urinary material available was small and its spectrum was considerably distorted by irrelevant absorption.

Extract C. This contained compounds liberated from conjugates on hydrolysis, and extractable at neutral pH.

6-Amino-4-nitro-*o*-cresol was separated by chromatography in benzene/acetic acid/water and benzene/*n*-butanol/ammonia and identified by comparison with an authentic spot, by its brown colour in 2N-NaOH, by its brownish reaction with HNO_2 and by its ultraviolet spectrum in 0·1N-NaOH after elution with ethanol. This metabolite, which could arise by the hydrolysis of *O*-conjugated 6-acetamido or 6-amino-4-nitro-*o*-cresol, accounted for 9·0–10·6% of the dose as determined spectroscopically in alkaline solution at 460 mμ. (cf. Fig. 3).

4-Amino-6-nitro-*o*-cresol appeared also to be present in very small amounts, since a spot was found with the same chromatographic properties as the authentic material in benzene/butanol/ammonia and benzene/acetic acid/water. It gave a violet colour with 2N-NaOH, reduced ammoniacal $AgNO_2$ and gave a red-violet colour in the diazo reaction, similar to those given by the authentic material. The amount present, however, was insufficient for spectroscopy.

In some experiments, this extract also appeared to contain some 3-amino-5-nitrosalicylic acid as indicated by separation of a spot with the same R_F value as the authentic material, a light brown colour with 2N-NaOH, a pink colour with HNO_2, a purple-brown colour with 1% $FeCl_3$ and an acid reaction to 1% aqueous bromocresol purple. Amounts were insufficient for spectroscopic examination.

The metabolites of 6-amino-4-nitro-o-cresol

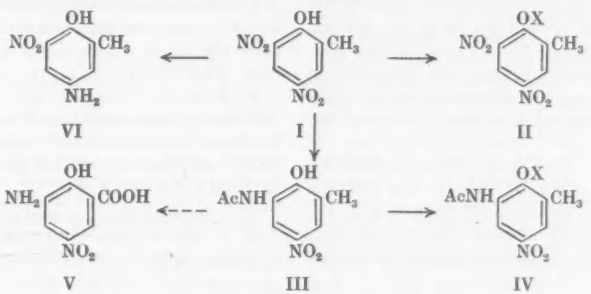
The cresol (0·6 g./kg.) was fed to a rabbit and a 24 hr. urine collected. Suitable small volumes (0·01–0·05 ml.) of the urine were chromatographed directly on the paper without previous extraction and the following compounds identified.

Unchanged 6-amino-4-nitro-*o*-cresol was identified by its chromatographic behaviour in benzene/acetic acid/water, *n*-butanol/acetic acid/water and benzene/*n*-butanol/water, by its brown colour in alkali and by its spectrum in 0·1N-NaOH after elution in 0·1N-NaOH. From the extinction at 460 mμ. it was calculated that in different experiments 7–18% of the dose was excreted unchanged over 3 days.

6-Acetamido-4-nitro-*o*-cresol was identified by its chromatographic behaviour in benzene/acetic acid/water, *n*-butanol/acetic acid/water and benzene/*n*-butanol/ammonia, by its yellow colour in alkali and by its spectrum in 0·1N-NaOH after elution from benzene/acetic acid/water chromatograms. From the value of the extinction at 425 mμ. it was calculated that, in different experiments, from 1 to 7% of a dose of 600 mg./kg. was excreted as this acetyl derivative.

The chromatograms also showed a large spot giving a yellow colour with 2N-NaOH. This was taken to be 6-amino-4-nitro-*o*-cresol glycuronide since it was present in the gum obtained by separating the glucuronides as the basic lead salts. It was not 6-acetamido-4-nitro-*o*-cresyl glucuronide (see below) since this does not give an immediate yellow colour with NaOH.

The metabolites of 6-acetamido-4-nitro-o-cresol. The urine (200 ml.) collected 24 hr. after feeding 2 g. of this compound was normal in appearance and gave a strong naphthoresorcinol reaction. The Benedict and diazo reactions were negative. Paper chromatography of the urine in benzene/acetic acid/water and *n*-butanol/acetic acid/water showed only two spots, one corresponding to the unchanged compound and the other to its glucuronide. The latter gave no immediate reaction with 2N-NaOH, but a yellow colour developed several hours after spraying the paper.



The glucuronic acid fraction of the urine was prepared through the basic lead salt and was obtained as a yellow syrup. This was neutralized with solid $NaHCO_3$, a little absolute ethanol was added and the mixture warmed. On cooling the hydrated *sodium salt* of *6-acetamido-4-nitro-o-cresyl glucuronide* crystallized. It was recrystallized from ethanol/water to give 300 mg. of pale yellow needles decomposing at 160–170° and showing $[\alpha]_D^{20°}$ – 29·2° (in water *c*, 2). (Found: C, 38·4; H, 5·8; N, 5·8; Na, 4·9. $C_{15}H_{17}O_{10}N_2Na.3{\cdot}5H_2O$ requires C, 38·2; H, 5·9; N, 5·9; Na, 4·9%. Light absorption: in 0·1N-HCl $\lambda\lambda_{max.}$ 240, 290, $\epsilon_{max.}$ 8900, 5500; in 0·1N-NaOH $\lambda\lambda_{max.}$ 245, 290, $\epsilon_{max.}$ 9200, 5500.)

DISCUSSION

Our experiments suggest that in 2 days after dosing with 20–30 mg./kg. of DNOC (I) rabbits excrete less than 20% of the dose as metabolites in the urine. About 5% of the dose is excreted as unchanged DNOC and a much smaller amount (about 1%) as conjugated DNOC (II). The main urinary metabolites are derivatives of 6-amino-4-nitro-*o*-cresol

which account for about 11–12 % of the dose. Some 1–1·5 % of the dose is excreted as 6-acetamido-4-nitro-*o*-cresol (III) and about 10 % as *O*-conjugates (IV) of this compound. Other compounds which seem to be excreted in small amounts appear to be 3-amino-5-nitro-salicylic acid (V) and derivatives of 4-amino-6-nitro-*o*-cresol (VI).

These results may be compared with those of Parker *et al.* (1951) in rabbits and dogs, where a 4–10 % excretion of DNOC was found, and those of Harvey *et al.* (1951) in man where a 1–2 % excretion was found. Both these groups of workers estimated DNOC in urine by the method of Parker (1949). This method probably estimates some 6-acetamido-4-nitro-*o*-cresol as well as DNOC, since the acetamido compound possesses appreciable absorption at 430 mμ., the wavelength used in Parker's method, and is also extractable by ethyl methyl ketone, the solvent used. The absence of the Derrien (diazo) test in DNOC urine noted by Parker *et al.* (1951) and the weak diazo tests found by us suggest that the bulk of the aminonitrocresols excreted are acetylated.

From our results it appears that the main detoxication of DNOC in rabbits is by reduction of the 6-nitro group and not by conjugation of the phenolic hydroxyl of DNOC alone. Much more free than conjugated DNOC is excreted. The main detoxication product, 6-acetamido-4-nitro-*o*-cresol, is a relatively innocuous substance and is at least 20 times less toxic than DNOC.

It is interesting to note that, in the intact rabbit, the 6-nitro group, which is ortho to the phenolic group, appears to be more readily reduced than the 4-nitro group of DNOC. Similar observations have been made in intact animals on 2:4-dinitrophenol (Parker, 1952) which is reduced in rats to 2-amino-4-nitrophenol, and on picric acid (Walko, 1901; Kozumi, 1914–15) which forms largely picramic acid (2-amino-4:6-dinitrophenol). Rat-liver suspensions, on the other hand, reduce 2:4-dinitrophenol to the other isomer, 4-amino-2-nitrophenol (Parker, 1952), which is apparently not formed in the intact animal.

SUMMARY

1. Using the techniques of paper chromatography and absorption spectroscopy, the urinary metabolites of 4:6-dinitro-*o*-cresol (DNOC) in rabbits have been examined.

2. After feeding doses of 20–30 mg./kg., less than 20 % of the dose could be accounted for as metabolites in the urine 2 days after dosing. Some 6–7 % was excreted as DNOC (about 5–6 % free and 1 % *O*-conjugated) and about 12 % as 6-acetamido-4-nitro-*o*-cresol (about 1·5 % free and 10–11 % *O*-conjugated). Traces of derivatives of 4-amino-6-nitro-*o*-cresol and 3-amino-5-nitro-salicylic acid were also excreted.

3. The conversion of DNOC into the 6-acetamido-4-nitrocresol resulted in a detoxication of DNOC since the latter compound is at least 20 times less toxic than DNOC.

4. 6-Amino-4-nitro-*o*-cresol and its *N*-acetyl derivative were also fed. The former was partly excreted unchanged and partly acetylated. The acetyl derivative formed a glucuronide which was isolated from the urine.

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Studies in Detoxication

49. THE METABOLISM OF BENZENE CONTAINING [$^{14}C_1$]BENZENE

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In earlier papers in this series (Porteous & Williams, 1949*a*, *b*; Garton & Williams, 1949; Parke & Williams, 1950, 1951*a*, *b*; 1952) attempts were made to account for the whole of an oral dose of benzene in rabbits. About 75% could be accounted for in gross terms of oxidized and unchanged benzene, and only a few of the metabolites excreted could be fairly accurately assessed. No reliable estimate of metabolites other than catechol, phenylmercapturic acid and muconic acid could be made. Using benzene labelled with one ^{14}C-atom, we have been able to make a reasonable balance sheet for the fate of a single dose of benzene and also to make accurate estimates of most of its metabolites.

EXPERIMENTAL

The ^{14}C-labelled benzene used was prepared by Dr H. S. Turner (Turner, 1951) and contained about 100 μc./ml. of radioactivity. It was administered to rabbits by stomach tube and diluted where necessary with unlabelled benzene which had been purified through the nickel cyanide ammonia clathrate compound (Evans, Ormrod, Goalby & Stavely, 1950).

After dosing, each rabbit was placed in a metabolism chamber (cf. Parke & Williams, 1950) which was swept with a continuous stream of air that was then bubbled through ethanol at about $-50°$ to trap unchanged benzene (Azouz, Parke & Williams, 1952*a*). In three out of four experiments arrangements were also made to collect CO_2, and in these cases the absorption train consisted of 2 Drechsel bottles (250 ml.) containing 50 g. of anhydrous magnesium perchlorate (Anhydrone) followed by 2 Drechsel bottles (50 ml.) immersed in a freezing mixture of acetone and solid CO_2 and containing 40 ml. of glass beads and 20 ml. ethanol to collect benzene. These were followed by the CO_2 absorbers consisting of 3 Drechsel bottles (250 ml.) fitted with sintered-glass dispersers and each containing 150 ml. of carbonate-free 5N-NaOH. CO_2 could be quantitatively recovered from the animal chamber by this train; thus the recovery of CO_2 liberated in the chamber from 25 g. of Na_2CO_3 by acid was 101% in 6 hr. During an experiment the ethanol and alkali in the absorption train were renewed every 6 hr. In the four experiments carried out, the absorption of unchanged benzene and CO_2 was continued for 30, 48, 48 and 70 hr., respectively, after dosing, after which times the animals were removed from the chamber.

In Exps. nos. 1 and 3 the animals were killed after 48 hr. and tissues analysed. In Exps. 2 and 4, the animals, after removal from the metabolism chamber, were placed in open cages so that their urine could be collected up to 7 days after dosing. The animals were then killed and the tissues of no. 4 analysed.

Measurement of radioactivity. All measurements were carried out on solid samples using an end-window counter tube, the background of which was approximately 9–11 counts/min. The specific activities were determined by comparison with known amounts of labelled benzene as $BaCO_3$ and sometimes as the nickel cyanide ammonia complex (see below) using the 'infinite thickness' technique (see Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949; Popják, 1950) with samples of 25 mg./sq.cm. of identical geometry prepared on aluminium planchettes (General Electric Co. Ltd.). A sample of about 2 sq.cm. containing 10^{-2} μc. of ^{14}C/mg. of substance gave approximately 7000 counts/min. Sufficient counts to give a standard error of $< \pm 2\%$ were taken. The variation in disk size gave an error of $\pm 5\%$. In the determination of the specific activity of the nickel cyanide ammonia complex the counting was carried out within 5 min. of the preparation of the sample on the planchette. This complex slowly loses its activity by loss of benzene from the surface layer and in an hour the counts/min. of an 'infinitely thick' sample dropped by about 2% at room temperature. This compound appears to be relatively stable in bulk. Permanent standards which showed no loss of activity over several months were prepared from [^{14}C]phenyl toluene-*p*-sulphonate. This ester was made from metabolic phenol isolated from the urine of rabbits receiving labelled benzene and standardized against $BaCO_3$ prepared from labelled benzene.

Urine and tissues. The total radioactivity eliminated in the urine was measured by evaporating to dryness, over P_2O_5 *in vacuo* at room temperature, 5 ml. urine mixed with 0·2 ml. N-NaOH. The residue was powdered and a weighed amount was submitted to wet combustion and the CO_2 evolved counted as $BaCO_3$ (see Calvin *et al.* 1949).

For the radioactivity of the tissues, 5 g. tissue were dispersed with 25 ml. 0·5N-NaOH in a Waring Blendor. The procedure for urine was then followed.

For the measurement of radioactivity present as benzene, the blood, urine or milk was mixed with an equal volume of N-NaOH, but tissues were dispersed as above. To 2·5 ml. of each mixture there were added 250 mg. of pure benzene as carrier and the mixture extracted with 5 ml. of light petroleum (b.p. 40–60°). The extract (2 ml.) was then shaken for 1 hr. with 15 ml. of an aqueous solution of ammonia nickel cyanide (prepared according to Evans *et al.* 1950). After keeping the mixture at 0° for a further 1 hr. the solid complex ($Ni(CN)_2$, NH_3, C_6H_6) was collected in a Gooch crucible, washed with water, ethanol and ether and dried at room temperature. The radioactivity of the solid complex was then determined as above within 5 min. of its

Table 1. *The occurrence of radioactivity in the tissues of rabbits receiving* [^{14}C]*benzene*

Exp. no.	1* (2 days)†		3 (2 days)†		4 (7 days)†	
	Wt. of tissue (g.)	% of dose present in tissue	Wt. of tissue (g.)	% of dose present in tissue	Wt. of tissue (g.)	% of dose present in tissue
Spinal cord	—	—	3	0 (0)‡	3·5	0
Brain	—	—	1·5	0 (0·01)	—	—
Blood	—	0·8 (3–4)‡	—	0 (0)	—	—
Intestine and contents	—	—	205	0·55 (0·1)	—	—
Stomach contents	—	—	55	0·15 (0·03)	—	—
Stomach	—	—	26	0·05	—	—
Spleen	—	—	1·1	0	1·2	0
Bone-marrow	—	—	Approx. 8	0	5	0·01
Heart	7	0 (0)	9	0	—	—
Lungs	38	0·2 (0)	5	0	—	—
Kidney	11	0·06 (0)	14·5	0·015	—	—
Liver	63	0·5 (0)	79	0·07	94	0·05
Testes	—	—	10	0·02	—	—
Voluntary muscle	800	9·3 (0)	700	1·6	1200	1·2
Involuntary muscle	200	1·5 (0)	—	—	—	—
Fat	200	2·6 (0)	180	1·5	—	—
Bile	—	—	1·5	0·01	—	—
Uterus	45*	0·9 (0)	—	—	—	—
Ovary	1·2*	0·03 (0)	—	—	—	—
Faeces	2·2	0·3 (0)	22	0·86	—	—
Total accounted for		16·19		4·8		

* The animal in this experiment was found to be diseased; the uterus was much enlarged and contained a chorion epithelioma. The specific activity of the uterus and ovary was twice that of the most radioactive normal tissue.

† Number of days after dosing with benzene when animal was killed.

‡ Figures in brackets give the amount found as free benzene.

preparation. The results for various tissues are given in Table 1, and Fig. 1 shows the elimination of radioactivity in the urine.

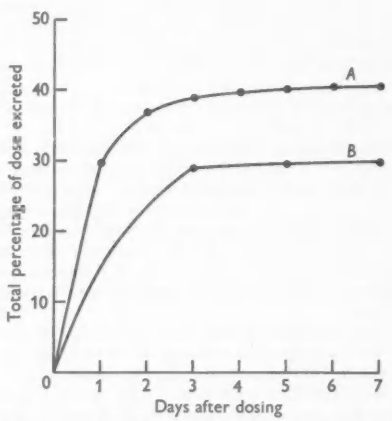

Fig. 1. The excretion of radioactivity in the urine of rabbits receiving ^{14}C-labelled benzene orally. *A*, Exp. 2 (dose of benzene, 0·34 g./kg.). *B*, Exp. 4 (dose of benzene, 0·4 g./kg.).

Carbon dioxide. Each 6 hr. fraction of the CO_2 absorbed in the absorption train (see above) was converted into $BaCO_3$ and the activity measured, as described by Calvin *et al.* (1949) (see Fig. 2 and Table 2). In Exp. 4 the radioactivity of the carbonate of the urine was determined. Sodium carbonate (0·5 g.) was added to 1/10 of the urine and this was acidified with 2N-H_2SO_4 and aerated. The CO_2 evolved was collected in N-NaOH and the carbonate converted to $BaCO_3$.

Benzene. The benzene eliminated in the expired air was determined spectrophotometrically (cf. Azouz *et al.* 1952*a*) using a Unicam spectrophotometer. Of the several absorption maxima of benzene in ethanol, that chosen for the present work was at 255 mμ. where ϵ was 240 (slit width of 0·4 mm.). The benzene was also estimated in one experiment by measuring its radioactivity. Unlabelled benzene (1 g.) was added to 5 ml. of the ethanolic solution of expired benzene and the nickel cyanide clathrate complex was prepared as above. The solid complex was centrifuged and repeatedly washed with 20% (w/w) ammonia solution until the supernatant was almost colourless and then with water, ethanol and finally ether. The solid was counted immediately. By this method, 46·5% of the dose was found, whereas the spectrophotometric method gave 48·4%. In the other experiments the spectrophotometric method only was used since it was inherently much more accurate than the counting method.

The figures of elimination of unchanged benzene and CO_2 in the expired air are given in Table 2. Fig. 2 shows graphically the elimination of $^{14}CO_2$, which begins at about 20 hr. after dosing. It is clear from the curves that the elimination of $^{14}CO_2$ had not ceased at the end of the experiment. In Exp. 1 the expired air was only examined for 30 hr. and the elimination of $^{14}CO_2$ had probably not begun.

Table 2. *The elimination of unchanged benzene and CO_2 in the expired air of rabbits receiving ^{14}C-labelled benzene orally*

Exp. no.	Dose of benzene (g./kg.)	Dose of ^{14}C μc./rabbit	Duration of exp. (hr.)	Unchanged benzene in expired air: Estimated spectrophotometrically (%)	Unchanged benzene in expired air: Estimated by radioactivity (%)	Respiratory CO_2 (% of dose)
1	0·34	123	30	45·9	—	0*
2	0·34	4·6	48	34·0	—	—
3	0·5	128	48	45·2	—	1·75
4	0·4	171	70	48·4	46·5	1·0

* The duration of the experiment was probably not long enough to detect radioactive CO_2; furthermore this animal was diseased (see footnote to Table 1).

Estimation of phenols

The urine from each rabbit was heated for 3 hr. under reflux with an equal volume of conc. HCl. This procedure hydrolysed glucuronides and ethereal sulphates. The various phenols were then isolated according to the following procedures.

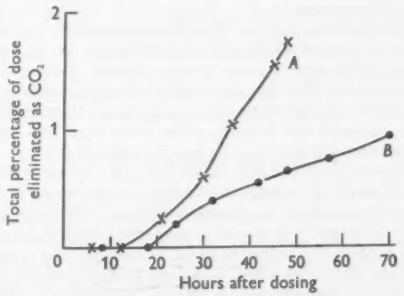

Fig. 2. The excretion of radioactive CO_2 in the expired air of rabbits receiving ^{14}C-labelled benzene orally. *A*, Exp. 3 (benzene, 0·5 g./kg.). *B*, Exp. 4 (benzene, 0·4 g./kg.).

Phenol. To 20 ml. of the hydrolysed urine 50 mg. of phenol were added. The solution was then steam distilled until 10 ml. of distillate had collected. To this was added 2 ml. of 40% (w/v) NaOH and the solution was then shaken for 20 min. with 0·25 g. of toluene-*p*-sulphonyl chloride in 10 ml. acetone. After 1 hr. the mixture was poured into water and the precipitated phenyl toluene-*p*-sulphonate collected and recrystallized from ethanol. The radioactivity of the toluenesulphonate was measured by the infinite thickness technique. It was recrystallized from ethanol until its specific activity became constant (m.p. and mixed m.p. 95°).

Catechol. Catechol (100 mg.) in a little water was added to 25 ml. of the hydrolysed urine and the solution continuously extracted with peroxide-free ether for 8 hr. The ether was evaporated (phenol is largely removed during this process) and the residue dissolved in a little water and the solution filtered. The catechol was then precipitated at pH 8 as the basic lead salt by the addition of aqueous basic lead acetate. The precipitate was collected and washed, and then suspended in water. H_2S was passed through the suspension to remove lead. After filtration, the catechol was extracted from the solution by continuous extraction with ether for 6 hr. After removal of the ether, the residue was dissolved in 5 ml. 2N-NaOH and shaken with 0·5 g. toluene-*p*-sulphonyl chloride in acetone (5 ml.). Catechol ditoluene-*p*-sulphonate was isolated by dilution with water and was recrystallized to constant specific activity from ethanol (m.p. and mixed m.p. 162°).

Quinol. In Exps. 1 and 2, quinol (200 mg.) was added to 20 ml. of the hydrolysed urine which was then continuously extracted with ether for 8 hr. After evaporation of the ether, the residue was dissolved in 10 ml. 2N-NaOH and quinol ditoluene-*p*-sulphonate prepared as for the catechol derivative. The ditoluenesulphonate was recrystallized first from ethyl acetate-ethanol and finally from ethanol until its specific activity was constant (m.p. and mixed m.p. 161°). In Exps. 3 and 4, the quinol was precipitated as the basic lead salt at pH 9·5 and the procedure for catechol followed.

Hydroxyquinol. Hydroxyquinol (200 mg.) was added to 20 ml. of the hydrolysed urine and the procedure of extraction and purification as the basic lead salt was carried out as for catechol. The final residue was then converted to the tritoluene-*p*-sulphonate. This ester was now further purified by dissolution in ethyl acetate and chromatography on a short alumina column. The ethyl acetate eluate was evaporated and the toluenesulphonate recrystallized from ethyl acetate-ethanol to constant specific activity (m.p. and mixed m.p. 106°).

Pyrogallol. This phenol (100 mg.) was added to 15 ml. of the hydrolysed urine and the procedure for hydroxyquinol followed exactly. On continued recrystallization from ethyl acetate-ethanol mixtures, then ethanol and finally aqueous ethanol the pyrogallol tritoluene-*p*-sulphonate (m.p. and mixed m.p. 139°) finally showed no radioactivity.

Resorcinol. Resorcinol (200 mg.) dissolved in a little water was added to 10 ml. of hydrolysed urine and recovered by continuous extraction with ether. The extracted resorcinol was purified through the basic lead salt and then converted to the dibenzoate (m.p. and mixed m.p. 117°). On repeated recrystallization from aqueous ethanol the dibenzoate never became completely free of radioactivity.

Since resorcinol has not been demonstrated colorimetrically or chromatographically in benzene urine, it was thought that the small amount of activity in the above dibenzoate might be due to adsorbed traces of other phenols. The phenols of benzene urine were therefore fractionated as follows.

To 1/10 of the total sample of hydrolysed urine, 1 g. of resorcinol in 10 ml. of water was added. The resorcinol was then extracted with ether in a continuous extractor for 8 hr. After evaporation of the ether, the residue was dissolved in 20 ml. water and filtered. Phenol (200 mg.), catechol (50 mg.) and quinol (50 mg.) were then added to the filtrate to effect the removal of the corresponding radioactive phenols by entrainment. The solution was steam-distilled to remove phenol. Catechol was removed from the solution as the insoluble lead salt by the addition of 20 ml. of saturated normal lead acetate solution at pH 6. After centrifuging to remove lead catecholate, the solution was adjusted to pH 9 with 2N-ammonia solution whereby the lead salt of resorcinol was precipitated. At pH 9, quinol, in the concentration used, just remains in solution. The lead salt of resorcinol was filtered off, well washed with water and dissolved in 2N-acetic acid and the precipitation of lead salts repeated. The purified salt of resorcinol was finally suspended in water and the lead removed with H_2S. Continuous ether extraction of the lead-free filtrate gave 0·6 g. of resorcinol, half of which was converted to the bis-3:5-dinitrobenzoate and the other half to the dibenzoate. After repeated recrystallization from acetone-ethanol the dinitrobenzoate (m.p. 200°) had a specific activity of 4 counts/min., whereas similar recrystallization of the dibenzoate (m.p. 116°) from ethanol also gave a constant count of 4/min. This specific activity corresponds to less than 0·1% of the dose but we never succeeded in obtaining an inactive resorcinol derivative. A qualitative examination of hydrolysed benzene urine by paper chromatography revealed the presence of phenol, catechol, quinol and hydroxyquinol but not of resorcinol, pyrogallol or phloroglucinol.

Our results on phenols are summarized in Table 3.

Diphenyl derivatives

When benzene is oxidized by free hydroxyl radicals, in addition to phenols, diphenyl is produced as a result of the combination of two phenyl radicals (Merz & Waters, 1949; Stein & Weiss, 1948, 1950). Since the phenols produced *in vivo* are similar to those produced by free-radical hydroxylation of benzene, the urine of rabbits receiving benzene was tested by the isotope dilution technique to see if it also contained diphenyl derivatives, namely diphenyl itself and *p*-diphenylylglucuronide. Tests were made for the glucuronide because diphenyl is known to be oxidized *in vivo* to 4-hydroxydiphenyl (Stroud, 1940) which is excreted as the glucuronide (Dodgson, Garton, Stubbs & Williams, 1948).

Diphenyl. A solution of diphenyl (250 mg.) in ether (10 ml.) was shaken for 0·5 hr. with 10 ml. of unhydrolysed [$^{14}C_1$]benzene urine to which 1 ml. 2N-NaOH had been added to keep any free phenols present in the aqueous phase. The ether layer was separated from the urine, which was then further extracted with 10 ml. ether. The mixed ether extracts were washed with 2N-NaOH then three times with water and finally dried over anhydrous Na_2SO_4. On evaporation of the ether, the residue of diphenyl, m.p. 60°, had a specific activity of 4 counts/min. After recrystallization from aqueous ethanol, the diphenyl had m.p. 66° (not depressing that of pure diphenyl, m.p. 69°) and no measurable radioactivity.

Diphenylylglucuronide. The glucuronide (100 mg.) dissolved in 1 ml. of saturated aqueous $NaHCO_3$ was added to 10 ml. of the urine and the mixture filtered. On acidification with 2N-HCl, the glucuronide was precipitated: it was purified by dissolution in aqueous $NaHCO_3$, treatment with charcoal and reprecipitation with acid, and twice recrystallized from water. It had no radioactivity.

Phenylmercapturic acid. Synthetic L-phenylmercapturic acid (0·4 g.) in a little 2N-Na_2CO_3 was added to 20 ml. urine. The solution was acidified with conc. HCl and kept at 0° overnight. The brown deposit was collected, washed with water and dissolved in 10 ml. N-$NaHCO_3$. The solution was extracted with ether to remove phenols, treated with charcoal, acidified with conc. HCl and kept overnight at 0°. This process of dissolution in bicarbonate and acidification was repeated twice and the product had m.p. 142°. Its

Table 3. *The elimination of phenols and other aromatic compounds in the urine of rabbits receiving ^{14}C-labelled benzene**

Exp. no.	1†	2	3	4		
Duration of experiment (days)	2	2	2	3	5	7
	% of dose					
Phenol	16·7	23·7	30·8	22·8	22·9	22·9
Catechol	1·2	2·1	2·6	2·8	2·9	2·9
Quinol	6·2	4·8	4·9	3·5	3·6	3·6
Hydroxyquinol	0·35	<0·1	0·4	0·3	0·32	0·32
Resorcinol	>0·3	>0·2	>0·2	—	—	—
Pyrogallol	0	—	—	0	0	0
Phenylmercapturic acid	0·7	—	0·4	0·4	0·4	0·4
Diphenyl	0	—	—	—	—	—
p-Diphenylylglucuronide	0	—	—	—	—	—
Sum of aromatic compounds plus *trans-trans*-muconic acid	27·25	31·6	40·4	30·9	31·2	31·2
Total radioactivity of urine	35·0	34·8 (41·7)‡	39·7	28·5	28·7	28·8

* Doses as for Table 3.
† The animal in this experiment was found to be diseased (see footnote to Table 2).
‡ Urine collected for 7 days after dosing.

Table 4. *Purification of phenylmercapturic acid from the urine of rabbits dosed with ^{14}C-labelled benzene*

Derivative	Purification procedure	^{14}C content: Counts/min.	^{14}C content: Counts/min. as phenylmercapturic acid
Phenylmercapturic acid	Three precipitations by acid	175	175
Phenylmercapturic acid	1st recrystallization from water	135	135
Phenylmercapturic acid	2nd recrystallization from water	131	131
Phenylmercuric mercaptide	Conversion to mercaptide	144	126
Thiophenyl *p*-nitrobenzoate	Crystallization from ethanol-chloroform	122	132

activity was then measured. It was then recrystallized to constant activity from water. As a criterion of purity, two derivatives were prepared. The acid (120 mg.) was hydrolysed to thiophenol by heating in 10 ml. N-NaOH for 1 hr. To 2 ml. of the solution was added 1 ml. ethanol and 0·6 ml. 10% $HgCl_2$. The precipitated phenylmercuric mercaptide (45 mg.) was filtered and washed with water. After drying its activity was determined. The thiophenol in the other 5 ml. of solution was continuously extracted with ether after acidification with 1 ml. conc. HCl. The ether extract was treated with 2 ml. pyridine and the ether allowed to evaporate at room temperature. Then 50 mg. *p*-nitrobenzoyl chloride was added and the mixture heated. The product was poured into water (10 ml.) and the precipitated thiophenol *p*-nitrobenzoate (25 mg.) washed with N-$NaHCO_3$. After recrystallization from ethanol-chloroform, it had m.p. and mixed m.p. 157° (Grillot, Levin, Green & Bashford, 1950). The results of a typical experiment are given in Table 4. The results are summarized in Table 3.

Open-chain acids of benzene urine

The problem of the formation of *trans-trans*-muconic acid from benzene *in vivo* has been recently discussed by us (Parke & Williams, 1952). Since radioactive benzene was available the opportunity arose to prove without doubt that *trans-trans*-muconic acid was a true metabolite of benzene. On injection of the muconic acids into rabbits, only 50–60% is excreted unchanged in the urine (Parke & Williams, 1952). Muconic acids therefore appear to be further metabolized and they could be intermediate in the conversion of benzene to carbon dioxide. If a muconic acid is an intermediate, it could by the process of β-oxidation, etc., be converted into fumaric, succinic and acetic acids and, since these acids are components of the tricarboxylic acid cycle, the final oxidation of benzene to carbon dioxide would appear to go via this cycle. On these grounds the urine of rabbits receiving labelled benzene was examined for these three acids. The urine was also examined for adipic acid, since muconic acid could be hydrogenated to this acid which could be an intermediate between the various isomers of muconic acid. Adipic acid fed to animals is partly excreted unchanged in the urine (Mori, 1918; Enders, 1941; Weitzel, 1947). Both oxalic and *meso*tartaric acids were found by Cook & Schoental (1950) to be the products of the oxidation of benzene with osmium tetroxide and hydrogen peroxide. The urine was therefore examined for these acids since osmium tetroxide oxidations seem to produce products analogous but not exactly similar to those produced in metabolic oxidations (cf. Schoental, 1950).

The muconic acids. (*a*) *The* trans-trans-*acid.* The acid (50 mg.) in 0·5 ml. 2N-Na_2CO_3 was added to 10 ml. urine, and then precipitated by acidification with 2N-HCl. The dark brown precipitated material was dissolved in 2N-$NaHCO_3$ and the solution treated with charcoal and filtered. The muconic acid was precipitated with acid and the process repeated. The final product (m.p. 300°) was washed with water, ethanol and ether and its radioactivity measured. It was then converted into its benzhydryl ester (Parke & Williams, 1952) which was recrystallized to constant activity from ethyl acetate (m.p. and mixed m.p. 190°). In Exps. 1–4 the urinary elimination of *trans-trans*-muconic acid represented 1·8, 1·0, 1·3 and 1·1% of the dose of benzene respectively.

(*b*) *The* cis-cis-*acid.* The acid (200 mg.) in 2 ml. 2N-Na_2CO_3 was added to 20 ml. urine and then precipitated with 2N-HCl. It was then purified by precipitation as for *trans-trans*-muconic acid. At this stage the acid had m.p. 188–189° and was markedly radioactive (e.g. 400 counts/min.). It was then converted into the benzhydryl ester which after two recrystallizations from ethyl acetate was inactive (m.p. and mixed m.p. 156°).

(*c*) *The* cis-trans-*acid.* The acid (100 mg.) in 1 ml. 2N-Na_2CO_3 was added to 20 ml. urine. After acidification to pH 2 with conc. HCl, the urine was continuously extracted with ether for 8 hr. The muconic acid was transferred from the ether to 3 × 10 ml. 2N-$NaHCO_3$. The bicarbonate extract was acidified and the muconic acid again extracted with ether. The ether was evaporated and the residual muconic acid converted into the benzhydryl ester. After a single recrystallization from ethanol, the ester had m.p. and mixed m.p. 142° and was inactive.

Acetic acid. Sodium acetate (250 mg.) was added to 10 ml. urine which was then acidified with 4 ml. 2N-HCl and continuously extracted with ether for 16 hr. The extracted acetic acid was taken into 5 ml. N-NaOH and the solution heated to remove ether, made just acid with 2N-HCl and then refluxed for 1 hr. with 0·65 g. of *p*-nitrobenzyl bromide. *p*-Nitrobenzyl acetate was precipitated by addition of water and cooling to 0°. After repeated recrystallization from ethanolethyl acetate, ethanol and aqueous ethanol the ester had m.p. 77° and had no appreciable radioactivity.

Succinic acid. Succinic acid (0·5 g.) in 2 ml. 2N-Na_2CO_3 was added to 10 ml. urine and after acidification was extracted with ether continuously for 16 hr. The residue after removal of the ether was dissolved in 2N-$NaHCO_3$ and the solution extracted twice with ether to remove phenols,

acidified with 2N-HNO_3 and 10 ml. N-$AgNO_3$ added. Silver succinate was precipitated by dropwise addition of 2N-ammonia solution. The precipitation process was twice repeated. Finally the silver succinate was dissolved in 0·5N-HNO_3 and the succinic acid extracted with ether (continuous, 8 hr.). After evaporation of the ether the acid was converted into the benzhydryl ester which after two recrystallizations from ethanol had no appreciable radioactivity (m.p. and mixed m.p. 142°).

Benzhydryl succinate was prepared by mixing succinic acid (0·1 g.) in 5 ml. methanol with diazodiphenylmethane (0·75 g.) in 5 ml. ether. After 6 hr. at room temperature, the solvents were evaporated. The residue (0·7 g.) washed with a little ether and recrystallized from methanol gave colourless needles, m.p. 143°. (Found: C, 79·0; H, 5·6. $C_{30}H_{26}O_4$ requires C, 80·0; H, 5·8%.)

Fumaric acid. Fumaric acid (1 g. in 10 ml. water) was added to 5% of the urine collected, followed by 20 ml. 10% $AgNO_3$. The mixture was adjusted to pH 8 with 2N-ammonia solution and the precipitate collected, washed with water, and then dissolved in 20 ml. of N-HNO_3 and the solution filtered to remove AgCl. The filtrate was treated with charcoal, filtered, and the silver fumarate precipitated with ammonia solution. It was collected, washed, dissolved in N-HNO_3 and reprecipitated with ammonia solution. At this stage the fumarate showed appreciable activity. The salt was therefore dissolved in 20 ml. N-HNO_3 and extracted continuously with ether. Concentration of the ethereal solution yielded fumaric acid which was still slightly active. On conversion to dimethyl fumarate (m.p. 101°), recrystallized from ethyl acetate-methanol, the radioactivity disappeared.

Adipic acid. The acid (0·5 g.) in 2 ml. 2N-Na_2CO_3 was added to 10 ml. of urine. Repeated acidification, ether extraction and solution in 2N-$NaHCO_3$ was carried out as for succinic acid. The final aqueous bicarbonate solution was acidified with 2N-H_2SO_4 and 10 ml. N-$CuSO_4$ added. Cupric adipate was then precipitated by the dropwise addition of 2N-ammonia solution. It was collected, washed with water, ethanol and ether, dissolved in 0·5N-H_2SO_4 and reprecipitated with 2N-ammonia solution. This was twice repeated and the final product dissolved in 0·5N-H_2SO_4 and the adipic acid extracted continuously (8 hr.) with ether. The extracted acid was converted into the dibenzhydryl ester which after two recrystallizations from ethanol had m.p. 90° and no significant radioactivity.

Benzhydryl adipate was prepared in the same way as the succinate. It formed colourless rhombs, m.p. 92°. (Found: C, 80·8; H, 6·6. $C_{32}H_{30}O_4$ requires C, 80·3; H, 6·3%.)

Oxalic acid. To 10 ml. of urine, 100 mg. of oxalic acid was added. The solution was slightly acidified with 2N-HCl, 10 ml. 10% $CaCl_2$ added and calcium oxalate precipitated by the dropwise addition of 2N-ammonia solution to the boiling solution. After 1 hr., the precipitate was filtered off, washed well with water, ethanol and ether. It was then dissolved in 10 ml. 2N-HCl, treated with charcoal and reprecipitated in boiling solution with 2N-ammonia solution. This was repeated and then the activity measured. In all cases the radioactivity was insignificant.

meso-*Tartaric acid.* *meso*-Tartaric acid (1 g.) in 10 ml. water was added to about 5% of the urine collected. The solution was neutralized with 2N-ammonia solution and then made just acid with 2N-acetic acid. On addition of 20 ml. 10% $CaCl_2$, calcium *meso*-tartrate was precipitated. After standing 24 hr. the precipitate was collected, washed with water and redissolved in 2N-HCl. After treatment with charcoal, the calcium salt was reprecipitated with ammonia solution. This process was repeated and finally the radioactivity disappeared.

DISCUSSION

Within the limits of error of the methods used, we feel that we can now account for most of an oral dose of benzene administered to rabbits. This is shown in the summarizing Table 5. Further details of the exact nature of the conjugates and the mechanism of the formation of muconic acid, however, have still to be worked out. Within 2–3 days of dosing, some 80% is eliminated from the body in the expired air, urine and faeces. The rest appears to remain in the body to be eliminated slowly in the urine, as shown in Exp. 4 where 28·5% of the dose had been eliminated in the urine in 3 days after dosing but only a further 0·3% was excreted during the succeeding 4 days, and in the expired air probably as carbon dioxide. The curves for the elimination of carbon dioxide (see Fig. 2) suggest that the elimination of benzene as carbon dioxide is far

Table 5. *The metabolic fate of a single oral dose of* ^{14}C*-labelled benzene in the rabbit, 2–3 days after dosing*

Nature of metabolite	% of dose (average)		
Benzene exhaled unchanged	43	44·5 in expired air	77 (79)‡
Respiratory CO_2	1·5		
Phenol*	23·5	32·6 in urine	
Catechol*	2·2		
Quinol*	4·8		
Hydroxyquinol*	0·3		
trans-trans-Muconic acid	1·3		
L-Phenylmercapturic acid	0·5		
(Urinary radioactivity)	(34·5)		
Metabolized benzene in tissues and faeces†	About 5–10 in body		
Total accounted for	84–89%		

* These phenols are excreted conjugated.
† About 0·5% of dose in faeces.
‡ Benzene in expired air + urinary radioactivity.

from complete after 2 days. That portion of the dose which remains in the body after 2 days appears to be present largely as metabolites. Only traces of free benzene were found and these were mainly in the stomach and intestinal contents. The radioactivity left in the body seemed to be generally distributed in most tissues and it appeared in greatest concentration in the bile, stomach, intestine, muscle and fat. Most of the radioactivity was found in the muscle and fat owing to their bulk. Exp. 4 (see Table 1) shows that over 1 % of the radioactivity remained in the animal after 7 days. The accurate assessment of the radioactivity remaining in the body is difficult owing to dilution by the bulk of the tissues.

More than half of the material eliminated from the body appears in the expired air, largely as unchanged benzene and to a small extent as respiratory carbon dioxide. In fact, the only new metabolite of benzene that these experiments have revealed is carbon dioxide. The formation of carbon dioxide was to be expected since it was already known that the benzene ring was broken *in vivo* to yield muconic acid. However, no more than 1–2 % of the dose appeared as carbon dioxide in 2 days, and no radioactivity was detected in the urinary carbonate, although carbon dioxide elimination was probably not complete in this time.

About 30 % of the dose is excreted in the urine as compounds containing the intact aromatic ring. The L-phenylmercapturic acid output in three experiments was 0·7, 0·4 and 0·4 % of the dose, which is somewhat lower than that (about 1 %) found by Parke & Williams (1951*b*) using non-isotopic methods. The major urinary metabolite of benzene has now been proved to be phenol which accounts for nearly one-quarter of the dose or about 70 % of the benzene metabolized and excreted in the urine. This phenol and the others are excreted almost entirely conjugated (Porteous & Williams, 1949*a*, *b*). Quinol is quantitatively the next major metabolite accounting for nearly 5 % of the dose and catechol, just over 2 %, the next. Hydroxyquinol is a minor metabolite (0·3 %). The urine was also tested for resorcinol and pyrogallol and although we were unable to eliminate the radioactivity completely from the carrier resorcinol, we feel, from other observations, that it and other phenols are not formed from benzene *in vivo*. At this point it is interesting to compare the outputs of quinol and catechol after feeding benzene with those obtained after feeding phenol. With benzene the ratio quinol/catechol is about 2, but with phenol the ratio is about 10 (Porteous & Williams (1949*a*) give the quinol output after phenol as 5 % of the dose, and Azouz, Parke & Williams (1952*b*) the catechol output as 0·6 %). This may suggest, as pointed out by Garton & Williams (1949), two routes from benzene to catechol, one via phenol and the other through an unknown intermediate.

The experiments with diphenyl and *p*-diphenylylglucuronide were carried out because of the similarity of orientation found in the biological and free-radical hydroxylation of benzene. The present experiments, however, show that diphenyl derivatives are probably not formed in the biological process, although diphenyl is found during the free-radical process in the presence of oxygen (Stein & Weiss, 1948, 1950).

Our experiments with the isomeric muconic acids now prove definitely that *trans-trans*-muconic acid is a metabolite of benzene. The results with these acids were very clear-cut and leave no doubt that *cis-cis*- and *cis-trans*-muconic acids are not excreted in the urine of rabbits dosed with benzene. They do not, however, throw any further light on why the *trans-trans*- is excreted rather than the expected *cis-cis*-isomer. In an earlier paper (Parke & Williams, 1952) we suggested that *cis-cis*-muconic acid need not be a precursor of *trans-trans*- during its formation from benzene. A reconsideration of the results of Bernhard & Gressly (1941), however, has caused us to doubt the validity of this suggestion. Bernhard & Gressly fed benzene containing 10·7 atoms % deuterium and isolated *trans-trans*-muconic acid containing 7·21 atoms % deuterium. The calculated deuterium content of a muconic acid containing 4 deuterium atoms would be $4/6 \times 10{\cdot}7 = 7{\cdot}13$ atoms % which agrees with the value found experimentally. This agreement suggests that *cis-cis*-muconic acid is first formed and is isomerized to the *trans-trans*-acid without the removal of the deuterium atoms. The formation of hydroxylated intermediates as suggested by us would have resulted in the removal of deuterium atoms, and a lower deuterium content would be expected in the isolated muconic acid.

Possible β-oxidation products of muconic acid are acetic and fumaric acids. Neither these nor succinic, oxalic, adipic and *meso*tartaric acids were found in the urine to possess radioactivity after [^{14}C]benzene feeding.

Further details of the metabolism of benzene are now being pursued.

SUMMARY

1. A study has been made of the fate in rabbits of an oral dose of benzene labelled in one carbon atom with ^{14}C.

2. In 2 days about 45 % of the dose is eliminated in the expired air, of which 43 % is unchanged benzene and about 1·5 % is carbon dioxide. Whereas the elimination of unchanged benzene is nearly complete in this time, that of carbon dioxide is not.

3. In the same time nearly 35 % of the benzene is eliminated as metabolites in the urine. These consist mainly of conjugated phenols. Phenol is the major metabolite accounting for about 23 % of the dose. The other phenols excreted are quinol (4·8 %), catechol (2·2 %) and hydroxyquinol (0·3 %). L-Phenylmercapturic acid accounts for 0·5 % of the dose.

4. The only open-chain acid which is found in the urine and is derived from benzene, is *trans-trans*-muconic acid (1·3 % of the dose). Radioactive *cis-cis*- and *cis-trans*-muconic acids, acetic, oxalic, adipic, succinic, fumaric and *meso*tartaric acids were not found in the urine. The carbonate of the urine contained no radioactivity.

5. Diphenyl and *p*-diphenylylglucuronide added to the radioactive urine were recovered devoid of activity, and hence these are not produced biologically from benzene.

6. The respiratory and urinary excretions account for nearly 80 % of the dose of benzene in 2–3 days after dosing, and the remainder appears to remain in the body to be eliminated slowly in the urine and probably in the expired air as carbon dioxide.

7. Two days after dosing, about 5 % of the administered radioactivity was found in the tissues generally. This occurred mainly as metabolites of benzene, and very little unchanged benzene was found after this time.

8. These experiments account for 80–90 % of the administered benzene.

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Biosynthesis of Milk-fat in the Rabbit from Acetate and Glucose. The Mode of Conversion of Carbohydrate into Fat

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The main object of the investigation described in this article was to study the mode of conversion of carbohydrate into fat. It will be shown that glucose is converted very effectively not only to fatty acids but also to glycerol. With the aid of acetate, pyruvate and glucose labelled with ^{14}C it has been possible to demonstrate that the conversion of glucose into fatty acids takes place after the breakdown of glucose to acetate, probably via pyruvate. The lactating rabbit was used as the experimental animal and the biosynthesis of glyceride fat studied in the mammary gland, because recent experience has shown that the mamma of a lactating animal is particularly suited for such purpose (cf. reviews by Folley, 1952; Popják, 1952*a*, *b*).

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In addition to the primary aim of the investigation quantitative data have been obtained which define with some degree of probability the percentage of milk fatty acids (especially of short-chain fatty acids) derived from acetate and glucose respectively. Some of the results have already been communicated in a preliminary form (French & Popják, 1951; Popják, 1952*b*, *c*).

EXPERIMENTAL

Rabbits (wt. 2·5 kg.) between the 12th and 16th days of lactation were used. Animals with a litter of four or more were preferred on account of their better yield of milk. The treatment of all experimental animals was the same unless stated otherwise. For 2–3 days before the experiment the mothers were separated from their litters in the morning and were milked 5–6 hr. later in order to make them accustomed to handling. For the night they were put back with the young.

For the actual experiment the mothers were again separated from the litters and, without preliminary milking, the test substance(s) administered. They were milked 6 hr. later and, when the experiment required, were killed by a dose of Nembutal and the mammary glands and the liver removed.

The comparison of acetate and carbohydrate as sources of milk fat was made in some experiments in the same animal. In such case [^{14}C]acetate was given first and the animal milked 6 hr. afterwards. [^{14}C]Starch or [^{14}C]glucose was administered 3–4 days later, when radioactivity was barely detectable any more in the milk fat, and the animal milked again 6 hr. afterwards. All animals were fed *ad lib.* before and during the experimental periods. This was essential in order not to interfere in any way with milk production and with the general metabolism of the animal. Ample evidence is now available to show that fasting fundamentally modifies lipid metabolism.

Milking. The animals were given intravenously 2–4 i.u. of oxytocin and were milked by applying intermittent suction of about 14 cm. of Hg to the nipples through the apparatus shown in Fig. 1. The funnel-shaped end of a male-urethral catheter served conveniently as the 'teat-cup'. The intermittent suction was made by closing and opening with the finger side arm *A* in Fig. 1. This apparatus proved far more flexible than an automatic milking machine, which was also tried, as it allowed a free variation of the rate of suction according to the 'let-down' of milk. The best yields of milk were obtained by applying intermittent suction at the rate of 30–40 times/min. at the beginning of milking and then, when the milk began to flow freely, by increasing the rate of suction to 80–100 times/min. During the periods of suction the 'teat-cup' was gently pulled so that not only suction, but also traction was applied to the nipples. The yield of milk varied a good deal from animal to animal; on the average about 25 ml. of milk could be obtained in less than half an hour, but some animals gave as little as 10 ml. or as much as 55 ml. The animals were held lightly on their backs by an assistant during milking; no restraint or anaesthetic was required.

Substances administered. These were: [*carboxy*-^{14}C]acetate and [*Me*-^{14}C]acetate (obtained from the Radiochemical Centre, Amersham, Bucks); [^{14}C]starch and [^{14}C]glucose prepared from tobacco leaves which were allowed to photosynthesize in an atmosphere of $^{14}CO_2$ (Porter & Martin, 1952). Samples of [1-^{14}C]glucose were kindly given by Dr R. Bentley and Dr K. Bloch. [α-^{14}C]Pyruvamide and [β-^{14}C]pyruvamide were synthesized as described by Anker (1948*a*); the pyruvate was freshly prepared before each experiment by the hydrolysis of pyruvamide with HCl followed by neutralization with NaOH. The labelled acetate and glucose were given in solutions, either by vein or by stomach tube and made isotonic with NaCl when necessary. The starch, in solution, was administered by stomach tube. Pyruvate was given always by vein.

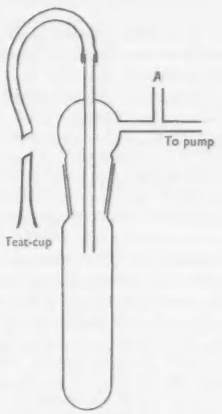

Fig. 1. Apparatus used for milking rabbits. Intermittent suction was applied to the nipples by closing and opening side arm *A* with the finger.

p-Aminobenzoic acid (1 g.) was dissolved in slight excess of N-NaOH, the pH adjusted to about 7 with HCl and the volume of the solution made up to 50 ml. with water. It was given by stomach tube.

Extraction of fat from the milk, mammary gland and liver. The milk was poured into 20 vol. of ethanol-ether mixture (3:1, v/v) and boiled for 5 min., the extract filtered hot and the residue re-extracted three times with 5 vol. of ethanol-ether. All extracts were combined.

The mammary gland, freed from connective-tissue fat as well as possible, was frozen with solid CO_2 and crushed in a chilled mortar as it was found very difficult to divide the gland finely by other means. The liver was minced for the extraction. The extraction of the tissues and fractionation of lipids and fatty acids were done as described previously (Popják & Beeckmans, 1950; Popják, French & Folley, 1951). Octanoic acid for chemical degradation (see later) was isolated from the water-insoluble steam-volatile fatty acids of milk and mammary-gland fat by vacuum distillation in a Podbielniak Heligrid-packed microdistillation column (Podbielniak Inc., Chicago, Ill., U.S.A.).

Preparation of lactose from milk. The ethanol-ether extract of the milk was evaporated to dryness under reduced pressure and the fat was extracted with light petroleum (b.p. 40–60°). The light-petroleum extract usually contained some finely suspended material, most of which was lactose and was separated by centrifugation. The residue in the

distillation flask, after the extraction with light petroleum, was almost pure lactose (often crystalline) and was dissolved in about 5 ml. of distilled water and added to the small amount of lactose separated from the light petroleum. This crude lactose solution was shaken three times with 3 ml. of ether and centrifuged each time; the ether was pipetted off leaving a thin pad of insoluble material on the surface of the aqueous solution which was filtered after the last extraction with ether and concentrated to a syrup on a hot-water bath. Boiling ethanol was added to the hot syrup until faint cloudiness appeared in the solution. Lactose crystallized out at 4° usually in 24 hr.; it was purified by crystallization from 60% (v/v) ethanol, washed with absolute ethanol and acetone and dried *in vacuo*.

Preparation of glycerol tribenzoate from glycerides. The method employed has been described previously (Popják, Glascock & Folley, 1952).

Extraction and degradation of p*-acetamidobenzoic acid from urine.* The acidified urine was extracted with ether in a continuous extractor for 24 hr. as described by Bloch & Rittenberg (1945). The purification of *p*-acetamidobenzoic acid by crystallization from hot water after treatment with charcoal, as described by these and other authors, was found, however, to involve very large losses on account of the strong adsorption on charcoal. Purification through the sodium salt was found far more satisfactory. The ethereal extract of the urine was evaporated to dryness and there were added to the residue 10 ml. of N-HCl which dissolved any free *p*-aminobenzoic acid together with a great deal of yellow pigment. The crude *p*-acetamidobenzoic acid was filtered off on a small Büchner funnel, washed with cold water, suspended in 20 ml. of water and dissolved by adding 0·1 N-NaOH to about pH 8. The solution was made up to 80 ml. with distilled water and made acid to pH 4 with HCl, when a dark-brown flocculent precipitate separated out. This was centrifuged off leaving a slightly coloured solution of *p*-acetamidobenzoic acid which was extracted immediately into ether and the ether evaporated off. The same process as above was repeated twice more. After the third purification the volume of the alkaline solution was kept down to 10 ml.; after acidification with HCl to pH 3–4, *p*-acetamidobenzoic acid began to crystallize almost immediately. The crystallization was completed overnight in the cold. The crystals were filtered off, washed with ice-cold distilled water and dried at 110°; m.p. 254–256°. From the urine (100–150 ml.) collected for 6 hr. after the administration of 1 g. of *p*-aminobenzoic acid usually 200–300 mg. of *p*-acetamidobenzoic acid were obtained.

For the separation and chemical degradation of the acetyl group a weighed amount of *p*-acetamidobenzoic acid was hydrolysed with 30% excess of ethanolic KOH on a boiling-water bath for 3 hr.; the ethanol was evaporated off and gradually replaced by water. After acidification with H_2SO_4 the acetic acid was separated by steam distillation. Usually 1 ml. of N-acetic acid was added to the mixture before distillation. The distillate was titrated with 0·1 N-LiOH and the solution evaporated to dryness. Silver acetate was prepared for radioactive counting from a small sample of the lithium acetate, the remainder being used for degradation by pyrolysis at 380–400° in a manner similar to that described for barium acetate by Calvin, Heidelberger, Reid, Tolbert & Yankwich (1949). The degradation of labelled lithium acetate by pyrolysis gives an error in that 1·2% of the activity of the carboxyl carbon appears in the CHI_3 (derived from the methyl carbon of acetone, hence from the methyl carbon of acetate), and 2% of the activity of the methyl carbon of acetate appears in the Li_2CO_3 (carboxyl carbon) of acetate. The values of radioactive counts were corrected for these errors. The CO_2 from the Li_2CO_3 was liberated with lactic acid *in vacuo*, trapped in $Ba(OH)_2$ and assayed for ^{14}C content as $BaCO_3$. The CHI_3 was oxidized to CO_2 *in vacuo* by the technique of Van Slyke, Plazin & Weisiger (1951) and the CO_2 collected as $BaCO_3$.

Partial degradation of octanoic acid. Carbon atoms 1 and 2 (carboxyl carbon and α-carbon) were obtained as acetic acid and carbon atoms 3–8 as caproic acid by a method previously described (Hunter & Popják, 1951). The acetic acid was further degraded by pyrolysis of lithium acetate. Carbon atoms 7 and 8 were also obtained as acetic acid by chromic acid oxidation of octanoic acid (Kuhn & Roth, 1933). The acetic acid was obtained from the oxidation mixture by steam distillation and was further purified by partition chromatography (stationary phase 0·5 N-H_2SO_4, moving phase $CHCl_3$; $CHCl_3$-butanol (9:1, v/v)). It was found that the oxidation product was not pure acetic acid, but a mixture of acetic (75–80%) and of propionic acid (20–25%). Only the pure acetic acid was used for further degradation to obtain carbon atoms 7 and 8.

Assay of ^{14}C. This was done on solid samples of infinite thickness as previously described (Popják, 1950).

RESULTS

Synthesis of fat in the mammary gland of lactating rabbits

The synthesis of fatty acids and of cholesterol in the mammary gland of non-lactating pregnant rabbits, at least 1 week before parturition, has already been demonstrated. This was shown by the incorporation of deuterium from the body water into these substances at a rate greater than that which occurred in the liver lipids (Popják & Beeckmans, 1950) and by the utilization of [^{14}C]acetate for both fatty-acid and cholesterol synthesis in the mammary gland (Popják, Folley & French, 1949; Popják & Beeckmans, 1950). It remained yet to be proved that this was also the case during lactation, although Folley & French (1950) have inferred from respiratory measurements made on tissue slices that the mamma of lactating rabbits also synthesizes fat from at least glucose.

It was known to us from other experiments on rabbits that the highest radioactivity in the plasma lipids occurred about 24 hr. after the injection of [^{14}C]acetate. It was argued, therefore, that if the specific radioactivity of the fatty acids and of cholesterol in milk and mammary gland were to exceed the radioactivity of the fatty acids and of cholesterol in the liver (this organ being the source of plasma lipids) much before the time at which the maximum activity of the plasma lipids was observed, proof of the occurrence of synthesis in the mammary gland would be obtained. A time of 6 hr. after the administration of a particular labelled substance was chosen as a convenient experimental point.

It should be mentioned that the specific activities of the fractions of milk and mammary-gland fat were found equal 6 hr. after administration of either labelled acetate, pyruvate or glucose. For this reason and in order to simplify presentation of data only one set of figures will be given in the tables either under the heading of 'mammary-gland fat' or 'milk fat'. In the former case it is implied that the milk fat was analysed together with the mammary-gland fat, and in the latter that only the milk fat was studied. Lactose was, however, obtained always from the extraction of milk alone.

The data in Table 1 show that the specific activities of the substances extracted from the mammary gland were appreciably greater than those obtained from the liver 6 hr. after the administration of either [*Me*-^{14}C]acetate or of [^{14}C]glucose. The two experiments are typical of several others which yielded essentially similar results. Fat synthesis from acetate and from carbohydrate in the mammary gland of lactating rabbits may therefore be taken as proved.

Comparison of acetate and carbohydrate as precursors of milk fat

It was thought necessary to make a comparison between acetate and carbohydrate as precursors of milk fat in the same experimental animal (cf. Experimental section) in order to determine whether or not either of these substances is used preferentially by the mammary gland. In Table 2 the results of two such experiments are recorded (see also Table 5) and show that the counting rates given by the fatty acids and by cholesterol were very similar after the administration of equal radioactive doses of acetate and of carbohydrate, i.e. the results do not suggest a preferential utilization of acetate over carbohydrate or vice versa for fatty-acid and cholesterol synthesis. However, the radioactivities of lactose and of glycerol indicate, as will be discussed later, that glucose acts as a direct source of glycerol, whereas acetate fulfils the same function indirectly only after its incorporation into carbohydrate. On this ground glucose might be regarded as a better precursor of 'fat' than acetate.

Mode of conversion of carbohydrate into fat. Breakdown of glucose to acetate

The fact that labelled glucose gave rise not only to labelled fatty acids but also to labelled cholesterol, suggested that glucose is broken down in the body to acetate. This was the more likely since it was shown by Bloch & Rittenberg (1944) that only those substances which gave rise to C_2 compounds were used for the biosynthesis of cholesterol. The only exception to this rule appears to be *iso*valeric acid (Zabin & Bloch, 1950).

We tested this idea by experiments in which [^{14}C]acetate or [^{14}C]glucose, together with *p*-aminobenzoic acid, were administered to normal and lactating rabbits. It is known that foreign amines, like *p*-aminobenzoic acid, sulphonamides and others, are excreted in the urine as the acetylated compounds (Harrow & Mazur, 1932) and that acetate is an effective acetylating agent for these substances (Bernhard, 1940). In particular, it is thought that *p*-aminobenzoic acid is acetylated only by acetate (Anker, 1948*b*). If, therefore, glucose is broken down in the body to acetate, then after the administration of [^{14}C]glucose together with *p*-amino-

Table 1. *Specific activity of glyceride fatty acids and of cholesterol in mammary gland and liver of lactating rabbits 6 hr. after administration of* [Me-^{14}C]*acetate and of* [^{14}C]*glucose*

Exp. no.		Volatile fatty acids: Soluble in water (10^{-3} μc.^{14}C/mg. C)	Volatile fatty acids: Insoluble in water (10^{-3} μc.^{14}C/mg. C)	Non-volatile fatty acids: Saturated (10^{-5} μc.^{14}C/mg. C)	Non-volatile fatty acids: Unsaturated (10^{-5} μc.^{14}C/mg. C)	Cholesterol (10^{-5} μc.^{14}C/mg. C)	Substance and dose administered
29	Mammary gland	2·30	2·35	23·00	45·00	26·00	[*Me*-^{14}C]Acetate 8·3 mg.; 100 μc.
	Liver	—	—	0·90	1·00	15·00	
31	Mammary gland	1·65	1·98	2·40	7·50	5·00	[^{14}C]Glucose 653 mg.; 91 μc.
	Liver	—	—	0·10	0·16	0·70	

Table 2. *Specific activities of milk constituents (counts/min./C in infinite thickness) from rabbits after administration of* [carboxy-^{14}C]*acetate or* [^{14}C]*carbohydrate*

Exp. no.	Labelled substance and dose administered	Lactose	Glycerol	Steam-volatile fatty acids: Soluble in water	Steam-volatile fatty acids: Insoluble in water	Non-volatile fatty acids	Cholesterol
17	Acetate (4·1 mg.; 50 μc.)	539	486	2880	2760	355	234
	Starch (37·3 mg.; 50 μc.)	11 760	13 332	2855	2740	276	372
18	Acetate (4·1 mg.; 50 μc.)	161	—	2068	2158	432	169
	Glucose* (357 mg.; 50 μc.)	5 560	3 883	1430	1520	324	299

* Milk collected during 12 hr., all others 6 hr.

Table 3. *Specific activity of the carbon of 'acetyl', liver fatty acids and liver cholesterol after the administration of* [carboxy-^{14}C]*acetate and of* [^{14}C]*glucose to normal rabbits for* 3 *days*

(The total dose of each substance was divided into six equal parts which were given to the animals by stomach tube twice daily; the total ^{14}C dose administered was 75 μc. with each substance.)

Substances administered	Specific activity (10^{-3} μc.^{14}C/mg. C) 'Acetyl' (I)	Fatty acids (II)	Cholesterol (III)	$\frac{(II)}{(I)} \times 100$	$\frac{(III)}{(I)} \times 100$
$CH_3.{}^{14}CO_2Na$	7·96	0·144	0·506	1·81	6·36
[^{14}C]Glucose	2·84	0·054	0·167	1·90	5·88

Table 4. *Specific activity of the carbon of 'acetyl' excreted with* p*-aminobenzoic acid, milk lactose and of glycerol prepared from glycerides of milk and liver of two lactating rabbits* 6 *hr. after administration of* [^{14}C]*glucose*

(In Exp. no. 31, 653 mg. of glucose uniformly labelled and containing a total of 91 μc. of ^{14}C were injected intravenously as a 5% solution. Immediately before the injection 1 g. of *p*-aminobenzoic acid (as the sodium salt) was given by stomach tube.

In Exp. no. 36, 10 mg. of labelled glucose, containing 30 μc. of ^{14}C and 1 g. of *p*-aminobenzoic acid (as the sodium salt) were given together by stomach tube.)

Exp. no.	Specific activity (10^{-3} μc.^{14}C/mg. C) Acetyl (I)	Lactose (II)	Glycerol From milk fat	Glycerol From liver fat	$\frac{(II)}{(I)} \times 100$
31	1·273	6·25	4·78	4·57	20·4
36	0·447	2·12	1·00	0·63	21·1

benzoic acid the animal should excrete [*Ac*-^{14}C]*p*-acetamidobenzoic acid.

Table 3 shows that the normal rabbit excretes radioactive acetyl groups after the administration of [^{14}C]glucose just as after [^{14}C]acetate. Moreover, the ratio of the specific activity of the fatty acids and of cholesterol in the liver to the specific activity of the acetyl groups excreted after the administration of [^{14}C]glucose was almost identical with that found after the administration of [^{14}C]acetate (see last two columns of Table 3). It is concluded, therefore, that in the rabbit glucose is converted to fatty acids and to cholesterol after being broken down to acetate.

The fraction of body acetate derived from glucose

It was of interest to determine the proportion of body acetate which is derived from glucose. This can be calculated if it is possible to compare, after the administration of [^{14}C]glucose and of *p*-aminobenzoic acid, the ^{14}C content of the excreted acetyl group with that of the glucose metabolized during the experiment (i.e. in this case with the ^{14}C content of the precursor of the acetyl group). Ideally, the specific activity of blood glucose should probably be used for such purpose, but for the preparation of glucose (as the phenylosazone) from blood at least 10 ml. samples are needed; however, the drawing of several samples of this size within a short time from a rabbit would very likely prejudice the experimental results. The lactating animal was thought, therefore, to be a good experimental subject for our purpose, because it secretes into the milk lactose which is derived mostly from blood glucose. It has been shown that after the administration of [^{14}C]-glucose to lactating rabbits, the specific activities of the glucose and galactose moieties of lactose were equal (French, Popják & Malpress, 1952). It seemed to us a reasonable assumption therefore that the specific activity of lactose would represent the average specific activity of blood glucose and hence of the glucose metabolized during the experimental period. In a few experiments glucosazone was prepared from the blood of animals which were given [^{14}C]glucose, [^{14}C]acetate, or [^{14}C]pyruvate. The specific activity of blood glucose was within 10% identical with that of lactose thus substantiating the above assumption.

Table 4 shows that 6 hr. after the administration of [^{14}C]glucose the specific activity of the acetyl C excreted during the experimental period in the urine with *p*-aminobenzoic acid was about 20% of the specific activity of lactose. Since lactose is secreted also continuously, its specific activity is an average for the experimental period just as is that of the acetyl C. It may be concluded, therefore, that about 20% of that part of the body's acetate, which was used for acetylation, was derived from carbohydrate in these animals. It should be emphasized that our animals were not fasted before the experi-

Table 5. *Specific activities (counts/min./C in infinite thickness) of fatty acids of milk glycerides and of cholesterol in rabbits after administration of substances labelled with* ^{14}C

(The total radioactive dose with each substance was 50 μc.)

Substance administered	Volatile fatty acids: Soluble in water	Volatile fatty acids: Insoluble in water	Non-volatile fatty acids	Cholesterol
$CH_3.{}^{14}COOH$	2068	2158	432	169
$^{14}CH_3.COOH$	2605	2825	413	299
$CH_3.{}^{14}CO.COOH$	2144	2601	283	175
$^{14}CH_2.CO.COOH$	2000	2160	300	128
[^{14}C]Starch	2125	2000	110	166
[^{14}C]Glucose	1430	1520	324	299
[^{14}C]Glucose	2260	2380	67	—

ment, and consequently the specific activity of liver glycogen could not be used as a measure of the glucose metabolized. We have experimental evidence to show that in a rabbit fed *ad lib.* it may require several days before the liver glycogen becomes equilibrated with blood glucose, and furthermore that the animal metabolizes first carbohydrate absorbed from the diet. For example, the specific activity of the liver glycogen in five experiments out of six was very much lower than that of milk lactose or that of the glycerol of liver triglycerides, which shows that glucose with a higher specific activity than that of liver glycogen was metabolized.

Pyruvate has been thought for a long time to be an intermediate in the conversion of carbohydrate to fatty acids, and since the most probable pathway of acetate formation from glucose seemed to be the oxidative decarboxylation of pyruvate, a further comparison was made between acetate, pyruvate and glucose as precursors of milk fatty acids. The results of such a comparison are shown in Table 5, from which it is evident that the amounts of ^{14}C incorporated from all three labelled substances into fatty acids and into cholesterol were essentially the same. Considering the fact that each experiment recorded in Table 5 was carried out on a different animal the radioactive counts show remarkably small variation. This might be purely fortuitous or it might mean that the metabolic pool of fatty-acid precursor became labelled to the same extent from the administered [^{14}C]pyruvate and [^{14}C]glucose as from [^{14}C]acetate. It was therefore necessary to compare the specific activity of the milk fatty acids with the specific activity of the excreted acetyl group after the administration of labelled acetate, pyruvate and glucose. While it is very uncertain to what extent the radioactivity of 'acetyl' C represents the radioactivity of acetate in the mammary gland, nevertheless the comparison may act as a useful guide to the approximate amount of the fatty acids synthesized in the mammary gland from acetate. The values shown in Table 6 indicate that in 6 hr. 30–70 % of the short-chain fatty acids (up to about C_{12}) might be derived from acetate provided that the specific activity of the excreted 'acetyl' C represents the specific activity of acetate in the mammary gland. Whether or not this assumption is correct cannot be determined at present. It is to be noted that the figures in Table 6 show a much greater variation than the figures in Table 5. This fact suggests to us that the radioactivity of the 'acetyl' C excreted in the urine does not really represent the radioactivity of the metabolically active acetate in the mammary gland. In the experiments in which [^{14}C]glucose was administered the radioactivity of the short-chain fatty acids was about 25 % of that of milk lactose (Table 7). This value can be interpreted with much greater confidence to mean that in 6 hr. 25 % of the short-chain fatty acids were derived from carbohydrate.

Table 6. *Specific activity of short-chain fatty acids in milk fat as a percentage of the specific activity of acetyl group excreted with* p*-aminobenzoic acid 6 hr. after administration of acetate, pyruvate and glucose labelled with* ^{14}C

Substance administered	Short-chain fatty acids: Soluble in water	Short-chain fatty acids: Insoluble in water
[*carboxy*-^{14}C]Acetate	51·2	55·6
[*Me*-^{14}C]Acetate	29·6	32·0
[^{14}C]Glucose	64·4	68·9
[1-^{14}C]Glucose	42·0	52·5
[α-^{14}C]Pyruvate	34·0	38·5
[β-^{14}C]Pyruvate	63·7	68·9

Table 7. *Specific activity of short-chain fatty acids in milk fat as a percentage of the specific activity of lactose 6 hr. after administration of* [^{14}C]*glucose to lactating rabbits*

Exp. no.	Short-chain fatty acids: Soluble in water	Short-chain fatty acids: Insoluble in water
16	26·3	21·8
17	24·2	23·3
18	25·7	27·4
31	26·3	31·6

Table 8. *Distribution of* ^{14}C *in milk octanoic acid and in 'acetyl' excreted in the urine after administration of substances labelled with* ^{14}C

Substance administered	Acetyl carbon atom		Octanoic acid					
			Carbon atoms 3–8 together (Determined value)	Carbon atoms 3–6 together (Calculated value)	Carbon atom no. (Determined values)			
	1	2			1	2	7	8
[*carboxy*-^{14}C]Acetate	>99·0	<1·0	50	50	>99	<1	>99	<1
[α-^{14}C]Pyruvate	96·5	3·5	50	50	96	4	96	4
[*Me*-^{14}C]Acetate	2·0	98·0	50	50	2	98	2	98
[β-^{14}]Pyruvate	3·7	96·3	50	50	4	96	4	96
[1-^{14}C]Glucose	3·6	96·4	50	50	5·8	94·2	5·8	94·2

Distribution of ^{14}C *in fatty-acid chain after administration of specifically labelled precursors*

Whether or not carbohydrate is converted into fatty acids through a C_2 compound derived from the decarboxylation of pyruvate, can be tested with specifically labelled substances. According to the known reactions of the glycolytic cycle glucose is broken down to pyruvate in such a way that carbon atoms 1 and 6 of glucose will become the β-carbon of pyruvate, and carbon atoms 3 and 4 will become the carboxyl carbon of pyruvate. Pyruvate resulting from the metabolism of [1-^{14}C]glucose should be labelled, therefore, in the β-carbon atom; on decarboxylation this pyruvate should yield acetate labelled in the α-carbon. Whether or not these reactions occur in the animal can be determined by comparing the position of the carbon label in a fatty acid or in the acetyl group excreted with a foreign amine after the administration of appropriately labelled acetate, pyruvate and glucose. The distribution of the label in a fatty acid is being used in this case as an indicator of metabolic reactions in the same way as the labelling of glucose has been employed in the study of intermediary metabolism (cf. Wood, 1948).

Octanoic acid was chosen as the 'indicator' because it is relatively abundant in the milk and mammary-gland fat of rabbits, and also because it is among the acids of the highest specific activity under our experimental conditions. The biological samples could be diluted thus several fold by inactive octanoic acid when necessary so that the chemical degradation could be carried out on samples of convenient size.

The results of degradations of the acetyl group excreted in the urine with *p*-aminobenzoic acid and of the partial degradations of octanoic acid of milk and mammary-gland fat after the administration of $CH_3.{}^{14}COOH$, $^{14}CH_3.COOH$, $CH_3.{}^{14}CO.COOH$, $^{14}CH_3.CO.COOH$, and of [1-^{14}C]glucose are shown in Table 8. The specific activities of the carbon atoms in this table are expressed in arbitrary units, the average specific activity of all the carbon atoms of the molecule together being taken as 50. The absolute values of the specific activities of the carbon atoms are, of course, irrelevant from the point of view of the present discussion. It can be seen from Table 8 that [*carboxy*-^{14}C]acetate and [α-^{14}C]pyruvate gave rise to fatty acids labelled in the odd-numbered carbon atoms, and [*Me*-^{14}C] acetate, [β-^{14}C]pyruvate and [1-^{14}C]glucose yielded fatty acids labelled mainly in the even carbon atoms. It may be concluded therefore that carbohydrate is converted to fatty acids by the overall reactions:

glucose → pyruvate → acetate → fatty acid.

The derivation of glyceride glycerol from carbohydrate

It was observed, in our experiments, that after the administration of [^{14}C]glucose (or starch) to a lactating animal, blood glucose and milk lactose had the highest specific activity among the various body constituents studied and that the specific activity of

Table 9. *Specific activity (counts/min./C in infinite thickness) of lactose and of neutral-fat glycerol in milk of lactating rabbits* 6 *hr. after administration of various substances labelled with* ^{14}C

Substance and dose administered	Specific activity of		$\frac{(II)}{(I)} \times 100$
	Lactose (I)	Glycerol (II)	
[^{14}C]Starch; 12 μc.	1 385	965	70·0
[^{14}C]Starch; 50 μc.	11 760	13 332	113·4
[^{14}C]Starch; 50 μc.	6 960	4 180	60·0
[^{14}C]Glucose; 50 μc.	5 560	3 883	70·0
[^{14}C]Glucose; 100 μc.	15 420	11 800	76·5
[*carboxy*-^{14}C]Acetate; 50 μc.	539	486	90·0
[*Me*-^{14}C]Acetate; 50 μc.	581	376	64·7
[α-^{14}C]Pyruvate; 50 μc.	510	476	93·5
[β-^{14}C]Pyruvate; 100 μc.	1 063	971	91·4

the glycerol of milk fat varied between 70 and 100% of that of lactose. Furthermore, even after the administration of labelled acetate or pyruvate—both of which are known to yield labelled glucose—the specific activity of glycerol varied again between 65 and 90% of that of lactose (Table 9). These observations can be explained only by assuming the rapid and direct formation of glycerol from carbohydrate in the animal (cf. Popják *et al.* 1952).

DISCUSSION

The biosynthesis of fat in the mammary gland

The experiments have proved that synthesis from small molecules within the mammary gland is of major importance in the origin of milk fat in the rabbit just as it was found to be in the lactating goat (Popják *et al.* 1951). The results showed a further similarity to those obtained on the ruminant in that the short-chain fatty acids had the highest specific activity after the administration of labelled acetate, pyruvate or glucose. This finding confirms the previous conclusion that the short-chain fatty acids in milk originate by a synthetic process and not by the degradation of long-chain acids.

Folley & French (1950) concluded, on the basis of respiratory measurements of tissue slices *in vitro*, that mammary-gland slices of rabbits utilized glucose but not acetate for fat synthesis. The equal ease with which fatty acids are synthesized both from acetate and from glucose in the mamma of the intact rabbit might be, therefore, surprising. However, Folley & French (1950) found that, in the presence of glucose, acetate was also used for fatty-acid synthesis. Of course, in the intact animal both substrates are readily available. Since glucose is converted into fatty acids after its breakdown to acetate, it seems probable that the differences observed in respect of utilization of acetate and glucose by tissue slices *in vitro*, when these two substrates are added separately to the incubation medium, are related to the energy-yielding mechanism required for the activation of acetate (cf. Popják, 1952*a*; Folley, 1952).

In the experiment on a lactating goat, already referred to, the data were insufficient for the calculation of the amounts of fatty acids synthesized from acetate in the udder. The only quantitative data obtained showed that in 6 hr. 50% of the injected acetate not oxidized was converted into milk fatty acids. In view of the controversy centring around the origin of short-chain fatty acids in milk fat (cf. Folley, 1949; Achaya & Hilditch, 1950; Hilditch, 1952), the quantitative data obtained in this investigation are of interest, although they are by no means clear-cut. There are three values to be reconciled with one another. The comparison of the specific activity of the acetyl carbon, excreted in the urine, with that of the short-chain fatty acids in the mammary-gland fat indicated that 30–70% of these acids might have been synthesized from acetate (1). The comparison of the radioactivity of milk lactose with that of the short-chain fatty acids after the administration of labelled glucose showed that about 25% of these fatty acids were formed from glucose (2). On the other hand, 20–21% of the body's acetate used for acetylation was also derived from glucose (3). Clearly, the quantities mentioned under (1), (2) and (3) are somewhat conflicting. However, conclusion (1) is based on the assumption that the radioactivity of the acetyl C excreted in the urine represents also the radioactivity of the acetate metabolized in the mammary gland. This is by no means certain, and in fact seems to us rather doubtful. If this assumption were correct, then, since about 20% of the acetyl group is derived from glucose, the amount of the short-chain fatty acids synthesized from carbohydrate should have been found to be between 6 and 14%. The higher value found (25%) and the conclusion that glucose is converted to fatty acids by way of acetate (see later), suggests very strongly that the specific activity of the acetate in the mammary gland was significantly lower than that of the acetate used in the acetylation of *p*-aminobenzoic acid. It seems therefore that we are underestimating the percentage of short-chain fatty acids synthesized from acetate.

Conversion of carbohydrate to fat

Conversion into fatty acids. The experimental results presented have shown that glucose is broken down to acetate and that in the liver the ratio of the specific activities of glyceride fatty acids and of cholesterol to the specific activity of the acetyl group excreted in the urine was very similar whether labelled glucose or labelled acetate were administered to the animals. Since the most probable site of acetylation of *p*-aminobenzoic acid is the liver, it is a reasonable assumption that the radioactivity of the acetyl group represents the radioactivity of the metabolically active acetate in the liver from which fatty acids and cholesterol are synthesized. It is concluded, therefore, that glucose is converted to fatty acids and to cholesterol via acetate.

The results obtained by the degradation of fatty acids and of the excreted acetyl support in every way this conclusion. It was found that [*Me*-^{14}C]-acetate, [β-^{14}C]pyruvate and [1-^{14}C]glucose all yielded fatty acids labelled in a similar way, about 95% of the isotope content being confined to the even-numbered carbon atoms of the fatty acid chain; [*carboxy*-^{14}C]acetate and [α-^{14}C]pyruvate, on the other hand, gave fatty acids labelled in the odd-numbered carbon atoms. Fatty acids formed from [2-^{14}C]glucose, which we have not been able to test as yet, should be labelled as the acids after the administration of [*carboxy*-^{14}C]acetate or of [α-^{14}C]-pyruvate. Our conclusion is, therefore, that glucose is broken down in the glycolytic cycle to pyruvate; the oxidative decarboxylation of the latter yields acetate, which forms then the building unit for the synthesis of fatty acid and of cholesterol. The various hypotheses of possible

mechanisms by which fatty acids with a chain of an even number of carbon atoms could be formed from pyruvate have been reviewed by Breusch (1952). Rittenberg & Bloch (1945) thought that the formation of acylpyruvic acids followed by decarboxylation might provide a more direct conversion of carbohydrate to fatty acids than the mechanism we suggest here. Anker (1948*b*) supported Rittenberg & Bloch (1945) in their view on the basis of finding in one strain of rats only very small amounts of isotope in *p*-acetamidobenzoic acid after the administration of pyruvate labelled in either the α- or β-carbon atom, although the acetyl group excreted with sulphanilamide and the fatty acids were highly labelled. In another strain of rats, however, radioactive acetyl group was excreted both with *p*-aminobenzoic acid and with sulphanilamide after the administration of α- or β-labelled pyruvate. These results of Anker (1948*b*) could be explained equally well on the assumption that in the two strains of rats the acetylation of the two amines took place at different sites rather than being indicative of a direct utilization of pyruvate for fatty-acid synthesis. In our animals the conversion of both pyruvate and of glucose to acetate was so great as judged by the acetylation of *p*-aminobenzoic acid that we have no reason to suspect that, in the rabbit, glucose or pyruvate act as precursors of fatty acids otherwise than by virtue of their previous breakdown to acetate.

The figures in Table 8 show that all the ^{14}C in the fatty acids (or in the acetyl group) was not confined entirely to the alternate carbon atoms after the administration of [*Me*-^{14}C]acetate, [β-^{14}C]pyruvate or of [1-^{14}C]glucose. The metabolism of pyruvate and acetate via the citric acid cycle provides a means for the rearrangement of the label in these substances. That this is not purely a speculative view was shown, for example, by the degradation of the glucose unit of lactose isolated from the milk of a rabbit after the administration of [1-^{14}C]glucose. The degradation, carried out by Dr H. G. Wood and to be published later, showed that significant amounts of radioactivity were present in carbon atoms 3, 4 and 2, 5 in addition to 1, 6.

The formation of glycerol from glucose. Our results give some new information on a hitherto neglected aspect of the biosynthesis of fat in animals, i.e. on the origin and turnover of glycerol. The results presented in this and in a previous paper (Popják *et al.* 1952) demonstrate for the first time the formation of glycerol from glucose in the animal organism. The specific activity of glycerol in the present experiments varied between 65 and 95% of that of milk lactose. Since it was found previously that the specific activity of the glucose and galactose units in lactose are equal after the administration of [^{14}C]glucose (French *et al.* 1952), we interpret our findings to mean that 65–95% of the milk and mammary-gland fat was renewed within the experimental period. On the basis of the arguments presented earlier it seems probable that about the same percentage of the short-chain fatty acids was also newly synthesized in the mammary gland. However, the glycerol was derived from the mixed glycerides containing all types of the fatty acids and it should be recalled that the specific activity of the long-chain acids was only about one-fifth to one-tenth of that of the short-chain fatty acids (cf. Tables 2 and 5). The nearly complete renewal of glycerol in the mammary gland within 6 hr. is therefore somewhat surprising. The only interpretation that can be given at present to this finding is that the rate of renewal of glycerol and of fatty acids in glycerides is not equal and that the glycerol in the mammary gland is being metabolized in addition to providing a structural part of fat. In a previous experiment on a lactating goat (Popják *et al.* 1951) the half-life of milk-glyceride fatty acids was calculated to be 4 hr. (cf. Popják, 1952*a*) and that of the glycerol 3 hr. (Popják *et al.* 1952). The disparity between the rate of renewal of glycerol and of fatty acids was apparent in our experiments to an even greater extent in liver fat. It appears therefore that the glycerol part of fat is metabolized by the body faster than the fatty acid part.

Taking all our results together it is evident that the conversion of carbohydrate into fat means not only the formation of fatty acids but of the entire molecule, including glycerol.

SUMMARY

1. The biosynthesis of fat was studied in lactating rabbits with the aid of acetate, pyruvate and glucose labelled with ^{14}C.

2. It has been shown that both short- and long-chain fatty acids and cholesterol are synthesized from acetate and from carbohydrate in the mamma of lactating rabbits.

3. It was estimated that in 6 hr. at least 30–70% of the short-chain acids originate from acetate, or about 25% from carbohydrate; only one-fifth to one-tenth of these amounts of the long-chain acids were, however, synthesized in the mammary gland from either of these sources.

4. Some 65–95% of the glycerol part of fat, derived from carbohydrate, was newly formed in the mammary gland in 6 hr. It is concluded that the glycerol part of fat is more rapidly metabolized by the animal than the fatty-acid part.

5. About 20% of the body's acetate used for acetylation of *p*-aminobenzoic acid was derived from glucose in fully fed lactating rabbits.

6. Octanoic acid from milk and mammary-gland fat and the acetyl group of *p*-acetamidobenzoic acid

were degraded chemically after the administration of [*carboxy*-^{14}C] and [*Me*-^{14}C]acetate, [α-^{14}C] and [β-^{14}C]pyruvate and of [1-^{14}C]glucose.

7. The results of degradations showed that the conversion of glucose to fatty acids proceeds by the overall reactions equivalent to glucose → pyruvate → acetate → fatty acid.

We express our gratitude to Dr R. Bentley and to Dr K. Bloch both of whom generously provided us with the samples of [1-^{14}C]glucose. The photosynthetic experiments with tobacco leaves were carried out by Dr Helen Porter at the Botany Department of Imperial College of Science and Technology, whom we thank for her co-operation. The skilful technical assistance of Miss Christin Connolly and Mr F. Gaebler are also gratefully acknowledged.

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Urinary Porphyrins in Lead-treated Rabbits

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New chromatographic methods of identifying and separating porphyrins (Nicholas & Rimington, 1949, 1951; Nicholas & Comfort, 1949; Nicholas, 1951; Chu, Green & Chu, 1951) have made possible the detection of previously undescribed porphyrins in biological material. The ether-soluble porphyrins found in the urine of men and of animals exposed to lead have been regarded until recently as consisting mainly, if not entirely, of coproporphyrin III. However, a tricarboxylic porphyrin has recently been found (Nicholas & Rimington, 1951) in the urine of a case of industrial lead poisoning and in a preparation of supposed coproporphyrin from the urine of lead-treated rabbits, as well as from other sources. Di-, penta-, hexa- and hepta-carboxylic porphyrins were found by the same workers in other pathological conditions. Weatherall & Comfort (1952) have also described tri- and tetra-carboxylic porphyrins, and have obtained evidence for the existence of a pentacarboxylic porphyrin in the urine of lead-poisoned rabbits, and have found similar porphyrins in the urine of normal rabbits. Kench, Lane & Varley (1952*a*, *b*) have found coproporphyrin I as well as coproporphyrin III, and an uncharacterized porphyrin in the urine of men suffering from lead poisoning. We have examined the urinary porphyrins of lead-treated rabbits in more detail and the results are described in this paper.

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METHODS

Extraction of ether-soluble porphyrins from urine

Rabbits were maintained on a porphyrin-producing intake of lead as already described by us (Weatherall & Comfort, 1952). Urine was separated from faeces by a grid of suitable mesh in the receiving area of the metabolism cages, collected without preservative and stored at 7°. Volumes of 0·5–2 l. were extracted by one of the two procedures described by Weatherall (1952), involving treatment with ether and acetic acid and subsequent transfer to N-HCl. A few samples were extracted by adsorbing the porphyrins on calcium phosphate (Sveinsson, Rimington & Barnes, 1949) and dissolving in N- or 3N-HCl. Pooled HCl solutions were neutralized with sodium acetate, using bromophenol blue as external indicator, and the porphyrins were taken into ether. The solutions in ether were washed with water and taken to dryness under reduced pressure. The free porphyrins were esterified overnight with methanolic HCl or H_2SO_4, transferred to $CHCl_3$, washed with water, 0·1M-Na_2CO_3, and water, and were dried before chromatography.

Chromatography on aluminium oxide of porphyrin esters

The methyl esters were dissolved in 1–5 ml. of benzene and chromatographed by Nicholas's (1951) method on grade IV aluminium oxide. The chromatogram was developed with increasing concentrations of $CHCl_3$ in benzene and finally with increasing concentrations of methanol in $CHCl_3$. Where ultraviolet fluoroscopy showed red fluorescent material not removed by methanol, the column was extruded, extracted for 36 hr. with 7N-HCl, and the extract filtered free of solid aluminium oxide.

Chromatography on paper of porphyrin esters

The method of Chu *et al.* (1951) was followed in detail, using kerosene b.p. 180°. Fractions obtained from the aluminium oxide column chromatograms were examined in this way, using methyl esters of deuteroporphyrin IX, coproporphyrin I and coproporphyrin III as markers. The modification of Kench *et al.* (1952*b*) was also used, in which the second solvent is run at right angles to the first.

Chromatography on paper of free porphyrins

The method of Nicholas & Rimington (1949, 1951), depending on partition between lutidine and water, was used. Porphyrin esters were saponified in 7N-HCl for 35 hr. at room temperature before chromatography. Details of technique have been described by Weatherall (1952).

RESULTS

Chromatography on aluminium oxide columns

In all, six batches of esterified porphyrins from lead-treated rabbits were examined. Each batch contained between 1 and 5 mg. of porphyrin, and represented the output of about 10–50 rabbit days. Thirty-two rabbits contributed to the various batches; no rabbit contributed to more than two of the batches. One batch of about 0·5 mg. of porphyrin from four normal rabbits has also been examined: in view of the large amount of starting material necessary, detailed studies of the porphyrin of normal rabbit urine are incomplete and will be reported later. The chromatograms of the material from lead-treated rabbits were similar, and usually showed five fluorescent bands (numbered throughout this paper from below upwards) (Fig. 1*a*). Band 1 was a fluorescent colourless trace completely eluted by 10% chloroform in benzene.

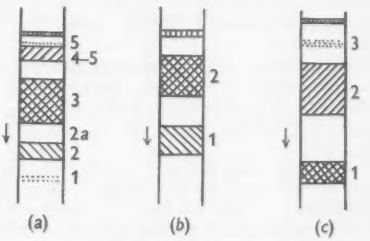

Fig. 1. Chromatograms on aluminium oxide of porphyrin methyl esters from urine of lead-treated rabbits, as developed with 10–20% $CHCl_3$ in benzene. (*a*) Preliminary run; (*b*) pool of predominantly 3-COOH porphyrins; (*c*) pool of high-COOH porphyrins. ▧, tricarboxylic porphyrins; ▩, tetracarboxylic porphyrins; ▨, penta- and hexa-carboxylic porphyrins; ▤, tri- and tetra-carboxylic porphyrins; ▥, mixed residue at top of column.

Band 2 was a substantial pink zone eluted by 10–15% chloroform. Band 3 was maroon in colour and contained the bulk of the porphyrin. It was also eluted by 10–15% chloroform, though more slowly than band 2, and it was found to consist mainly of coproporphyrin. Bands 4 and 5 were commonly fused. Band 4 ran as a pink zone and 5 as a fluorescent, usually colourless, band close behind it. A pigmented but non-fluorescent zone, pink or violet, probably consisting of bile-pigment esters, occasionally appeared between bands 2 and 3 (2*a*). The concentration of chloroform necessary to elute the various bands differed slightly in different runs, and the figures given are approximate. In one run the coproporphyrin moved exceptionally readily and no separation of antecedent bands was obtained.

The large eluted fractions were taken to dryness, dissolved in a few drops of benzene or chloroform and diluted with an equal volume of light petroleum. They were left at 5° to crystallize. Crystals were obtained on several occasions from band 3, and once from band 5 (sample 465). Some of their properties are shown in Table 1. Most of the crystalline samples behaved as coproporphyrin III on lutidine and on kerosene paper chromatography, though often they were not free from other porphyrins. Sample 465 had a melting point (146°, remelt 168°,

Table 1. *Urinary porphyrins of lead-poisoned rabbits*

(Melting points and paper chromatography of crystalline methyl esters. Sample 420 was an unchromatographed methyl ester. All the other samples were obtained from band 3 of alumina chromatograms, except 465, which came from band 5 of the chromatogram which had already yielded 463. Sample 434*b* was a second crop obtained on concentrating the mother liquor from 434*a*.)

Sample no.	M.p. (uncorr.) (°)	Appearance	Porphyrins detected on paper chromatogram: Lutidine/water system, after hydrolysis	Porphyrins detected on paper chromatogram: Chu method, methyl esters
420	138	—	Tetra- and penta-carboxylic	—
434*a*	146, remelt 164	Needles, typical of copro. III	Tetra-, trace penta- or hexa-carboxylic	Copro. III
434*b*	146	Ill-formed needles	Tri-, tetra-, penta-carboxylic	—
463	142	—	Tetracarboxylic	Copro. III
465	146, remelt 168	—	Tetracarboxylic	Not copro. I or III (see Table 2)
501	120	Rosettes. Not homogeneous	Tetracarboxylic	Three spots—copro. III and two substances with lower R_F values
511	134, remelt 140	Well-formed needles	Tetra-, penta- or hexa-carboxylic	Copro. III

uncorr.) like that of coproporphyrin III, but behaved differently on kerosene paper chromatography: it is further discussed below.

Samples of about 2 μg. were taken from the eluted fractions, and from the mother liquors from which crystals had been obtained. These samples were hydrolysed and chromatographed on lutidine papers to determine the carboxyl number of the porphyrins present. The fractions usually yielded two or more spots. On the whole the early eluates contained porphyrins with high R_F values and the later eluates contained those with lower values. Porphyrins running parallel to standard coproporphyrin were present in nearly all the eluates at this stage. The mother liquors from which porphyrins had been crystallized still contained appreciable amounts of red fluorescent material, and the paper chromatograms suggested that this remaining material was largely composed of tri-, penta- and hexa-carboxyl porphyrins.

The single batch of porphyrins extracted from normal, untreated rabbits' urine showed on aluminium oxide chromatography bands 1, 2, 3 (predominant) and one or more further bands which migrated more slowly than coproporphyrin. The various components appeared to be present in much the same relative proportions as in the extracts of the urine of lead-treated animals, though the quantities were much less.

In the further investigation of the material from lead-treated rabbits, the eluted fractions which were too small to crystallize, or which failed to crystallize, and the mother liquors were combined in groups according to their predominant components and were chromatographed again on aluminium oxide columns. This second series of chromatograms resembled the preliminary runs in giving rise to several fractions, but the proportions differed, as expected, from the composition of the starting material.

Apparently corresponding fractions from these runs were combined as before and a third series of solid chromatograms was run on aluminium oxide columns. Three runs were made, with pools rich in esters of porphyrins with three, four, and five or more carboxyls respectively. The tricarboxylic porphyrin pool (Fig. 1*b*) yielded two bands which separated well. The first band was eluted with 5% chloroform (sample 571) and on hydrolysis yielded only tricarboxylic porphyrins (R_F on lutidine chromatography, 0·77; comparable standard values are given in Table 2), while the second band was eluted with 10% chloroform and yielded tetracarboxylic porphyrins on hydrolysis (lutidine R_F, 0·64). The tetracarboxylic porphyrin pool ran as a single band eluted by 10% chloroform. The polycarboxylic porphyrin pool (Fig. 1*c*) divided into three bands of which the first two appeared well separated. The first fraction was eluted with 10% chloroform and on hydrolysis contained mainly tetracarboxylic porphyrins (lutidine R_F, 0·64) with a trace of pentacarboxylic material (R_F, 0·44). The second band was eluted by 20% chloroform (sample 592) and contained only hexacarboxylic porphyrin (R_F, 0·33). The third band was eluted by 30% chloroform (sample 593), and, rather surprisingly, on lutidine chromatography of a hydrolysed portion appeared to contain tri- and tetra-carboxylic porphyrins (R_F, 0·76 and 0·62).

As the amounts of material available were decreasing and the atypical porphyrins were proving difficult to separate completely, it was

Table 2. *Chromatography of urinary porphyrins from lead-treated rabbits*

(The figures given are the mean of three or less observations, the range of four to ten observations and the mean and standard deviation of more than ten observations.)

Porphyrin	Elution order from Al_2O_3	Paper R_F values: Free porphyrin (Lutidine/water)	Porphyrin methyl ester (1, kerosene/chloroform and 2, propanol/kerosene): One dimension (Solvents run successively over paper in same direction)	Two dimensions: Solvent 1	Two dimensions: Solvent 2
Standards					
Deuteroporphyrin IX	—	0·84±0·04 (S.D.)	0·41±0·02 (S.D.)	0·00	0·53
Mesoporphyrin IX	—	—	0·89*	—	—
Protoporphyrin IX	—	—	0·84*	—	—
Coproporphyrin I	—	0·62±0·04 (S.D.)	0·60±0·03 (S.D.)	0·31–0·64	0·05–0·15
Coproporphyrin III	—	—	0·77±0·03 (S.D.)	0·58–0·64	0·64–0·68
Urophorphyrin I	—	0·03	0·17	0·22	0·01
From Pb urine					
Coproporphyrin III	3	0·56–0·64	0·72–0·82	0·58–0·63	0·57–0·69
Porphyrin *A*	1	0·82–0·91	0·96	—	—
Porphyrin *B*	2	0·70–0·79	0·87	0·74	0·76
Porphyrin *C*			0·67	0·60	0·52
Porphyrin *D*	5	0·55–0·62	0·34–0·45	0·09–0·26	0·34–0·49
Porphyrin *E*	4+5	0·27–0·38	0·67–0·73	0·50–0·60	0·56–0·66
Porphyrin *F*			0·41–0·46	0·0 –0·13	0·24–0·36
Porphyrin *G*	5	0·76	0·51	0·39–0·44	0·46

* Figures given by Chu *et al.* (1951).

decided to get as much further information as possible about the purer fractions already obtained, rather than to embark on extensive further extractions of rabbits' urine in order to obtain additional material.

Elution of the residue at the top of the column with methanol, or its hydrolysis with 7N-hydrochloric acid, always yielded a mixture similar to the substances eluted from the column, and it did not therefore appear that selective loss of any particular fractions was occurring in this way.

Kerosene paper chromatography

Fractions which ran homogeneously on aluminium oxide columns and on lutidine papers were shown by kerosene paper chromatography to be non-homogeneous, and it was possible to recognize several porphyrins which occurred repeatedly in our material and which did not correspond to any of the standards examined. The regions of the two-dimensional chromatograms in which the standard and the unknown porphyrins appeared are shown in Fig. 2, and a provisional interpretation of the findings is discussed below. In order to exclude artifacts, portions of paper containing porphyrin esters separated by this method have been cut out, extracted with acetone and run as before. The fractions have run homogeneously with R_F values comparable to those observed in the first run, and have shown no tendency to divide again.

DISCUSSION

Nicholas (1951) has shown that the elution order of porphyrin esters chromatographed by her technique on grade IV aluminium oxide agrees with the number of esterified carboxyl groups, and Nicholas & Rimington (1949, 1951) have found an inverse linear relation between the number of carboxyl

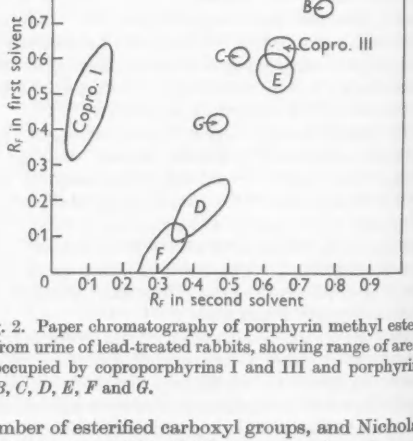

Fig. 2. Paper chromatography of porphyrin methyl esters from urine of lead-treated rabbits, showing range of areas occupied by coproporphyrins I and III and porphyrins *B*, *C*, *D*, *E*, *F* and *G*.

groups in a free porphyrin and its R_F values on chromatography on paper with lutidine and water. Our own results with deuteroporphyrin, coproporphyrin and uroporphyrin (Table 2) support this linear relation, and give us grounds for attributing carboxyl numbers to the various fractions examined. With certain exceptions, all the porphyrins which we have obtained from the urine of lead-treated rabbits are consistently identified by both methods. The lowest band (1), eluted from the aluminium oxide column with 5–10% chloroform, is a minute trace, sometimes too small even for identification by paper chromatography, but it has been twice identified after hydrolysis as a dicarboxylic porphyrin. Band 2 consists mainly of tricarboxylic porphyrin esters. Band 3, containing the bulk of the material, yields largely coproporphyrin, but in the preliminary chromatograms carried with it several other constituents in small amounts. Bands 4 and 5 contain a mixture of penta- and hexa-carboxylic porphyrin esters in varying amounts. However, lower carboxyl fractions sometimes appear in these final bands. In one preliminary run a tetracarboxylic porphyrin ester (465) was obtained from band 5 in sufficient amounts to crystallize. Also, the final band (593) of the pool of high-carboxyl esters gave both tri- and tetra-carboxylic porphyrins on hydrolysis. These tri- and tetra-carboxylic porphyrin esters did not behave on kerosene chromatography like the tri- and tetra-carboxylic esters which came off the aluminium oxide column at the expected time, and appear to be different entities. They are discussed below.

The majority of samples obtained from bands 4 and 5 gave free porphyrins with R_F values on lutidine papers of about 0·3. If the relation between R_F and number of carboxyls is strictly linear, this indicates a predominance of hexacarboxylic porphyrins. Occasionally spots with R_F about 0·45–0·50 were found. These spots would correspond well with a pentacarboxylic porphyrin and support the hypothesis that the main constituent of the high-carboxyl fraction contains six carboxyl groups. Porphyrins extracted from the urine, adsorbed on talc, eluted and run on lutidine papers have given R_F values about 0·35–0·40. We have previously reported observations indicating that a pentacarboxylic porphyrin is the main constituent of the high-carboxylic fraction (Weatherall & Comfort, 1952), but in the light of the present observations on more highly purified material we regard this interpretation as doubtful.

Kerosene chromatograms of the porphyrin methyl esters show that most of the fractions obtained by alumina chromatography are not homogeneous, even when the corresponding free porphyrins behave as single substances on lutidine chromatography. From the behaviour of the esters we incline to the view that the unknown tricarboxylic and hexacarboxylic porphyrins may exist in two isomeric series. If our chromatographic separations all correspond to structural differences, it is necessary to postulate no less than six or possibly seven porphyrin types other than coproporphyrin III, and perhaps I, in our material. The distinguishable porphyrins which appear to be entities are here cited by letters, and their properties are given in Table 2.

Porphyrin *A* corresponds to band 1 from the aluminium oxide column. It behaves as a dicarboxylic porphyrin on lutidine chromatography. One sample only was examined by the method of Chu *et al.* (1951). It ran with an R_F of 0·96, which is close to the value obtained by Chu *et al.* with mesoporphyrin IX and protoporphyrin IX. It may also correspond with the unknown porphyrin reported in the urine of human lead-poisoned subjects by Kench *et al.* (1952*b*). However, in our material, the possibility that it might arise from faecal contamination of urine specimens, though unlikely, is not wholly excluded.

The fraction eluted by 5 or 10% chloroform (band 2) contained tricarboxylic porphyrins. The purest samples (e.g. 571) ran as a single spot on lutidine papers, but as two, of roughly equal intensity, in one- and two-dimensional kerosene chromatograms (porphyrins *B* and *C*). Porphyrin *C* had an R_F value by this method very close to that of coproporphyrin III, but no tetracarboxylic material was detectable by Nicholas's method. It seems likely that *B* and *C* are distinct tricarboxylic porphyrins and the possibility exists that they are, in fact, position isomers of series I and III.

The coproporphyrin band (3) almost alone gave crystals. The crystallized material generally contained small traces of tri- and penta- or hexacarboxylic porphyrins on hydrolysis and lutidine chromatography but ran as a single spot corresponding to coproporphyrin III by the method of Chu *et al.* (1951). This behaviour is not unexpected, as the esters of porphyrin *C* and one of the hexacarboxylic porphyrins (*E*, below) have R_F values very close to those of coproporphyrin III.

Evidence of the presence of coproporphyrin I was scanty. Two mother liquors from which coproporphyrin III had crystallized contained a fraction running at $R_F = 0{\cdot}58$–$0{\cdot}63$ on one-dimensional papers by the method of Chu *et al.*, which agreed well with the behaviour of known coproporphyrin I run on the same strip: but the apparently greater solubility of this fraction is not typical of the series I isomer. No unequivocal coproporphyrin I was isolated in the crystalline state. The discrepancy between our finding and that of Kench *et al.* (1952*a*, *b*) might be expected from the far higher proportion of coproporphyrin III normally ex-

creted in rabbit urine as compared with human (Schwartz & Zagaria, 1951).

A third, presumably tetracarboxylic, porphyrin (*D*) was distinguished on kerosene chromatograms (sample no. 465). It was eluted from alumina with unusual difficulty, requiring 100% chloroform for full removal. Crystals obtained from benzene-light petroleum solution had a melting point 146° (remelt 168°, uncorr.) compatible with coproporphyrin III. It ran in lutidine water as a tetracarboxylic porphyrin. On two-dimensional paper chromatography, trace spots corresponding to the positions of coproporphyrins I and III were just detectable, but the bulk of the sample always behaved quite differently (R_F about 0·17/0·41) from coproporphyrin markers, and both on one-dimensional kerosene chromatography and on the column its behaviour was suggestive of a higher number of carboxyl groups than was found in any other substance isolated from our material. No other homogeneous sample of *D* was obtained, but there was evidence of a similar substance in the last fraction obtained by elution with 30% chloroform from an alumina chromatogram of the pooled high-carboxylic fractions. This sample (593) ran on hydrolysis mainly as a tetracarboxylic porphyrin with a trace of a tricarboxylic porphyrin. The ester ran on kerosene papers mainly like *D* with a smaller fraction at R_F 0·51 in one dimension and 0·44/0·46 in two. All these values are lower than those of *B* and *C*. This would suggest that there may be a tricarboxylic porphyrin (*G*) related to these porphyrins much as *D* is related to coproporphyrin. In view of the fact that alumina may cause partial saponification of porphyrin esters (Rimington, personal communication) the possibility that *D* and *G* are column artifacts requires further investigation.

The main hexacarboxylic fraction (e.g. 592) appears, like the tricarboxylic fraction, to consist of two components, one moving rather like coproporphyrin III on two-dimensional kerosene papers (fraction *E*) and one much more slowly (fraction *F*). Fraction *E* is differentiated from coproporphyrin III both by the behaviour of the hydrolysed porphyrin on lutidine papers and also by a consistently slightly lower R_F on one-dimensional kerosene papers.

The physiological significance of this large array of related substances is difficult to assess especially in view of the finding of one of us (Weatherall, 1952) that little if any of the ether-extractable porphyrin is present as such at the moment of excretion. The relative proportions of the porphyrins containing various numbers of carboxyl groups seem to differ little and there is no evidence that lead intoxication does more than provoke a general proportionate increase. Its effect on isomer distribution compared with the normal has not been studied by us, but we find very little if any coproporphyrin I in the lead-treated rabbit.

SUMMARY

1. The porphyrins present in the urine of lead-treated rabbits have been examined chromatographically.

2. The chief porphyrin present was coproporphyrin III. Coproporphyrin I was not convincingly demonstrable in any of the material, and if present constituted a very small fraction of the total.

3. Evidence was obtained for the presence also of porphyrins containing two, three, six and perhaps five carboxyl groups. It is suggested that the tri- and hexa-carboxylic porphyrins exist in two position-isomeric forms.

4. Evidence was also obtained for the presence of another tetracarboxylic porphyrin not identical with coproporphyrin I or III.

We are indebted to Prof. C. Rimington for frequent advice and for samples of standard porphyrins and to Miss H. Moore for much technical assistance. The expenses of the work were partly defrayed by a grant to one of us (M.W.) from the Medical Research Council. During part of the investigation one of us (A.C.) was supported as a research student by the Nuffield Foundation.

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The Radiation Chemistry of Ethanol and Diphosphopyridine Nucleotide and its Bearing on Dehydrogenase Action

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One of the most striking features of radiation chemistry is that the products are often those which would be produced by biological systems acting on the same substrate (Keller & Weiss, 1951; Weiss & Scholes, 1951; Rigg, Scholes & Weiss, 1952). An example is the oxidation of ethanol to acetaldehyde. This reaction has been induced by 27 MeV. α-particles (McDonell, 1951) and can also be carried out by liver and yeast alcohol dehydrogenases in the presence of diphosphopyridine nucleotide (DPN). It is tempting to suppose that the mechanism of such reactions may bear some resemblance, especially as changes induced by ionizing radiations can often be explained by free-radical mechanisms, and such mechanisms have also been suggested for the action of certain enzymes (Waters, 1943, 1948).

It was thought that a study of the action of γ-radiation on ethanol and DPN might give results relevant to the action of the alcohol dehydrogenases and, especially, might show whether chain reactions of the type suggested by Waters (1943, 1948) can take place.

EXPERIMENTAL

Irradiation arrangements. These were somewhat similar to those described in a previous paper (Swallow, 1952). Two sources of ^{60}Co γ-radiation of strength 100 mc. and 430 mc. were used, and also a 250 mg. source of radium. In order to obtain reproducible results, all quantitative irradiations were performed with solutions (1 ml.) irradiated in flat-bottomed tubes 1 cm. in diameter and 9·25 cm. high, fitted with greased ground-glass stoppers and immersed in a thermostat at 20±0·1°. When the irradiation tubes were at a certain distance from the 100 mc. ^{60}Co source, the dose rate was calculated from the previous measurement (Swallow, 1952) to be 400 r./hr., allowance having been made for decay of the source (half-life, 5·3 yr.). This was the dose rate for the results shown in Fig. 1. With other arrangements of sources and irradiation vessels the dose was estimated by measuring the amount of acetaldehyde produced from ethanol by irradiation, and comparing this with the amount produced by irradiation at the measured dose rate. The results shown in Figs. 2–4 and the results showing the effect of DPN on acetaldehyde yield and the effect of de-aeration on yield were at a dose rate of 725 r./hr.

Chemicals. Ethanol was obtained from the Distillers Company Ltd. and was stated to be 96·1% (v/v) ethanol and to contain only 0·0005% (w/w) aldehydes. As sodium pyrophosphate of A.R. purity was not obtainable, a commercial grade was recrystallized before use. HCl was redistilled. Water for solutions to be irradiated was distilled from alkaline permanganate. Preliminary experiments were performed with commercial DPN (L. Light and Co.) and later experiments with 55% pure DPN, kindly supplied by the Biochemistry Department of the University of Cambridge. The DPN used for the actual experiments described in this paper was from the Sigma Chemical Company, U.S.A., and was 90% pure. Other chemicals were of A.R. purity.

Deoxygenation of solutions. The method for deoxygenation and saturation with O_2-free N_2 or H_2 has been described previously (Swallow, 1952). It was necessary to make allowance for the decrease in ethanol concentration during deoxygenation. It was calculated that after the deoxygenation of 2 ml. of solution containing 3·0 m-moles ethanol, the remaining 1 ml. of solution would contain approx. 0·5 m-mole.

Measurement of acetaldehyde. The method is fundamentally that of Barker & Summerson (1941), originally intended for the measurement of lactic acid. It consists of the colorimetric measurement of the purple colour produced when *p*-hydroxydiphenyl reacts with acetaldehyde in conc. H_2SO_4. It was found that H_2O_2 (which is a possible radiation product) interferes when ethanol is present, and DPN also interferes in the presence of ethanol. The modification adopted eliminates both sources of interference. Other aldehydes interfere, but are not present in irradiated solutions.

A stream of N_2 is bubbled through the aqueous acetaldehyde solution and acetaldehyde is condensed in a test tube containing a mixture of 6 ml. conc. H_2SO_4, 0·9 ml. water and 0·05 ml. copper sulphate solution (4% $CuSO_4.5H_2O$). The aqueous solution is maintained at 24° and the condensing solution at about −20°. Bubbling is continued for 20 min., by which time all acetaldehyde has been removed from the aqueous solution and most of it collected in the H_2SO_4. This is allowed to warm to room temperature and 0·1 ml. of a *p*-hydroxydiphenyl solution (1·5 g./100 ml. 0·125 N-NaOH) is added as close to the surface of the liquid as possible. The test tube is well shaken to disperse the precipitated reagent through the solution, and after standing for at least 1 hr. it is heated in boiling water for 90 sec. After addition of 3 ml. conc. H_2SO_4 the intensity of the purple colour is measured with a colorimeter at about 560 mμ. A Hilger Biochem Absorptiometer was used for this purpose. The intensity is proportional to the amount of acetaldehyde within the

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range 0–24 μg. In order to obtain an absolute value for the amount of acetaldehyde in an irradiated solution, it is necessary to calibrate with known quantities of acetaldehyde on the same day as the unknown is measured. This was the procedure adopted for the points on Figs. 1 and 2, which are considered to be accurate to within $\pm 10\%$. When it is desired only to compare the yields from several irradiations it is not necessary to calibrate, provided all measurements are made on the same day.

Ultraviolet absorption curves. The curves shown on Figs. 3 and 4 were obtained by diluting the irradiated solution (1 ml.) with 3 ml. water and measuring the optical density with a Unicam photoelectric quartz spectrophotometer SP500. Distilled water was used as a blank. The author is indebted to Dr A. S. Jones for permission to use the instrument.

RESULTS

Production of acetaldehyde

A 1% aqueous solution of ethanol (6 ml.) was irradiated with a dose of approximately 2×10^6 r., and the aldehyde formed characterized by preparation of the 2:4-dinitrophenylhydrazone. The melting point was 164–165°. The melting point of authentic acetaldehyde 2:4-dinitrophenylhydrazone prepared by the same method was 165° and the melting point of a mixture of the two products also 165°, thus proving that the only aldehyde formed by irradiation is acetaldehyde.

An attempt was made to test for 2:3-butylene glycol in irradiated solutions. If the presence of this substance could be demonstrated it would be evidence of the existence of C_2H_4OH radicals in the system. The periodic acid method as given by Shriner & Fuson (1948) is interfered with by hydrogen peroxide and gave no positive results. There does not appear to be any method which would unambiguously detect quantities as small as those expected in the system and so, rather than develop a special method, the approach was abandoned.

The irradiations described above were of unbuffered aqueous ethanol, but in order to obtain results relevant to the alcohol dehydrogenases it was decided to irradiate under the same conditions of pH, type of buffer and temperature as are required for the action of these enzymes. All quantitative results on both ethanol and DPN were obtained with solutions made up in 0·01 M-sodium pyrophosphate adjusted to pH 7·8 with HCl, and were at 20°.

It was shown that the production of acetaldehyde from 0·5 M-ethanol in the presence of dissolved air is proportional to dose up to 66 000 r. (Fig. 1). From these results it was calculated that the yield of acetaldehyde under these conditions is 5·4 molecules/100 eV. absorbed.

The effect of deoxygenation is to decrease the yield of acetaldehyde to about 1·8 molecules/100 eV. It was thought possible that this might have been due to the concentration of ethanol in the de-aerated solution not being exactly 0·5 M (the calculation of this concentration being only approximate); the effect of ethanol concentration on yield was therefore investigated for air-saturated solutions. The result is shown on Fig. 2. It is apparent that the yield is not greatly dependent on concentration, and the decrease in yield on de-aeration cannot be explained by a change in ethanol concentration, and must therefore be due to the removal of oxygen.

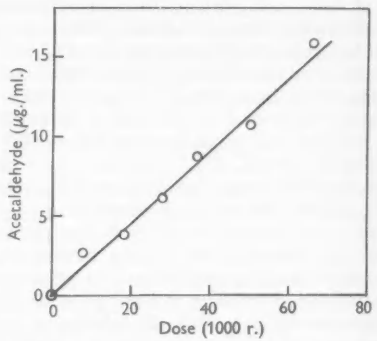


Fig. 1. The effect of γ-ray dose on the yield of acetaldehyde from 0·5 M-ethanol.

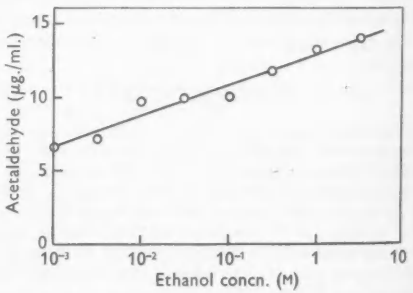


Fig. 2. Oxidation of ethanol to acetaldehyde by γ-radiation. Effect of ethanol concentration on yield (dose 52 000 r.).

The addition of DPN to 0·5 M-ethanol had little effect on the yield of acetaldehyde in the presence of air. DPN was neutralized with sodium hydroxide before making up solutions which were given a dose of approximately 30 000 r. The yield of acetaldehyde from 0·5 M-ethanol alone was 6·6 μg. and when the DPN concentration was $2{\cdot}7 \times 10^{-3}$ M the yield was 7·0 μg. When the DPN concentrations were $2{\cdot}7 \times 10^{-4}$ M and $2{\cdot}7 \times 10^{-5}$ M the yields were 7·2 μg. and 6·7 μg., respectively.

DPN had a much greater effect on yield of acetaldehyde in the absence of air. With a dose of about 30 000 r. the yield for ethanol alone was 2·2 μg. and with $2{\cdot}7 \times 10^{-3}$M-DPN the yield increased to 6·6 μg. With $2{\cdot}7 \times 10^{-4}$M- and $2{\cdot}7 \times 10^{-5}$M-DPN the yields were 7·2 and 4·0 μg., respectively.

It was thought possible that the increased yield in the presence of DPN might be due to the production of acetaldehyde from DPN itself. That this is not the case was shown by irradiating $5{\cdot}3 \times 10^{-4}$M-DPN solutions with a dose of about 30 000 r. Neither in the presence nor in the absence of oxygen was more than a trace of acetaldehyde produced, and this may have been due to ethanol impurity in the DPN.

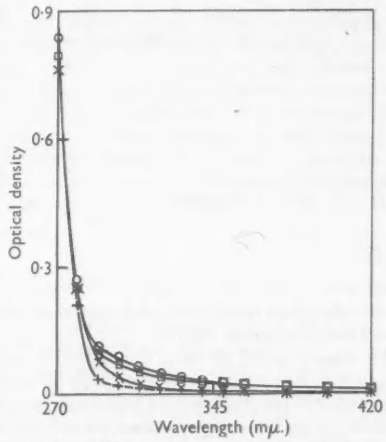


Fig. 3. Absorption curves of DPN after irradiation with a dose of 120 000 r. ×—×, Air-saturated solution; ○—○, oxygen-free solution; □—□, solution saturated with hydrogen; +—+, air-saturated solution containing 0·5M-ethanol and (identical curves) DPN with no irradiation.

Production of reduced DPN

Oxidized DPN (DPN^+) has an absorption band at about 260 mμ. and the reduced form (DPNH) has in addition an absorption band at 340 mμ. Racker (1950) used the absorption at 340 mμ. as a measure of the amount of DPNH present in mixtures of the oxidized and reduced form. It was decided to measure the ultraviolet absorption curve of irradiated $2{\cdot}7 \times 10^{-4}$M-DPN^+ solutions in the region 270–420 mμ., in order to see whether DPNH is formed. Results are shown in Figs. 3 and 4.

It can be seen (Fig. 3) that the absorption curve of DPN^+ irradiated with a dose of 120 000 r. in the presence of dissolved air is very little different from the curve of non-irradiated DPN^+, and the presence of 0·5M-ethanol completely protects it even from this small effect of irradiation. In the absence of air and ethanol no band due to DPNH appears when $2{\cdot}7 \times 10^{-4}$M-DPN^+ is irradiated with a dose of 120 000 r. Neither is DPNH formed when $2{\cdot}7 \times 10^{-4}$M-DPN^+ is irradiated in the presence of hydrogen with a dose of 120 000 r. All four irradiations were repeated with a dose of 30 000 r.: even smaller effects were observed.

When a DPN^+ solution containing 0·5M-ethanol is irradiated in the absence of dissolved oxygen with a dose of about 30 000 r., the characteristic absorption band at 340 mμ. appears (Fig. 4). Longer irradiation (60 000 r.) produces some increase in absorption at 340 mμ. but absorption increases at about 290–300 mμ. With still further

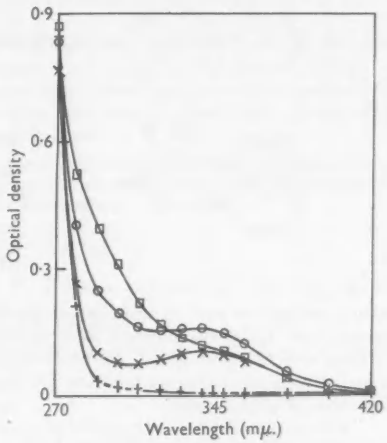


Fig. 4. Absorption curves of oxygen-free DPN solutions irradiated in the presence of 0·5M-ethanol. +—+, No irradiation; ×—×, dose 30 000 r.; ○—○, dose 60 000 r.; □—□, dose 120 000 r.

irradiation (120 000 r.) the characteristic absorption at 340 mμ. disappears and the absorption at 290–300 mμ. increases still further. These irradiations show that DPNH is the first product formed, and this itself appears to be further affected by radiation with the production of a substance absorbing at 290–300 mμ.

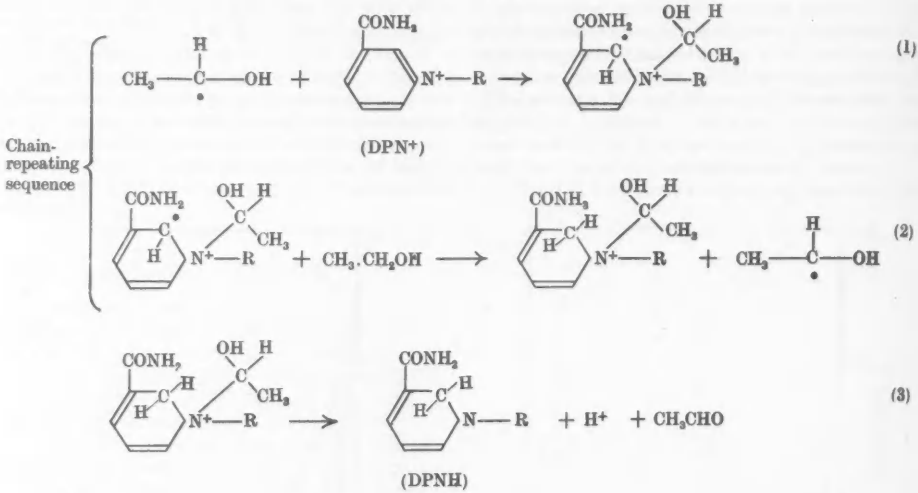
DISCUSSION

Waters (1943, 1948) has suggested free-radical equations to explain the action of certain enzymes. In the particular case of the alcohol dehydrogenases, the following reactions (p. 256) are an important part of one mechanism put forward.

These equations have so far little experimental support, but it can be seen that if the free C_2H_4OH radical could be produced under the same conditions of pH, temperature and concentration of reactants as are required for enzyme action, then it might be

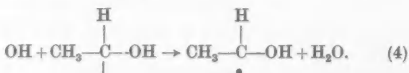
possible to show experimentally whether or not the above sequence of reactions can take place. In this investigation ionizing radiations were used as a means of producing the C_2H_4OH radical.

The results given in this paper show that in the presence of dissolved air the yield of acetaldehyde is almost unchanged, and DPNH does not appear. From this it may be concluded that these reactions



cannot take place under the conditions required for alcohol dehydrogenase action.

Ionizing radiations act on aqueous solutions by producing free hydroxyl radicals and hydrogen atoms from water (Weiss, 1944). In the presence of dissolved ethanol the following reaction, (4), would produce the C_2H_4OH radical:



The results obtained by McDonell (1951), in which 2:3-butylene glycol was one of the main products of irradiating pure ethanol with α-particles, show that in that case the radical C_2H_4OH is a transient reaction product, and that the C—H bonds in ethanol are more easily broken than the O—H bond. If it had been possible to demonstrate the existence of 2:3-butylene glycol in irradiated aqueous solutions this would have been strong evidence for the existence of the C_2H_4OH radical under these conditions, but this was not possible, and so the existence of the radical remains only a probability.

If the reactions represented by equations (1)–(3) can occur in aqueous solution at pH 7·8 and at 20°, then the irradiation of an aqueous solution of ethanol containing DPN^+ should result in a larger yield of acetaldehyde than in the absence of DPN^+ and should also result in the formation of DPNH.

The reason may be that the C_2H_4OH radical forms a peroxide with dissolved oxygen (Dewhurst, 1951) and this peroxide cannot take part in a chain reaction. In agreement with this, it was found that the irradiation of oxygen-free solutions gives quite different results. In this case the yield of acetaldehyde in the presence of $2{\cdot}7 \times 10^{-4}$ M-DPN^+ is about three times the yield in the absence of DPN^+, and DPNH is formed. However, the yield of acetaldehyde is only 5·9 molecules/100 eV. absorbed, which is not high enough to be proof of a chain reaction. An alternative explanation of these results, not involving the reactions (1)–(3), could be that the DPN^+ is reduced by free hydrogen atoms. If this were so, the hydrogen atoms would not be available for recombination with OH or C_2H_4OH radicals and so the observed increase in acetaldehyde yield could also be explained. However, it is clear from Fig. 3 that (*a*) the irradiation of DPN^+ without ethanol and in the absence of air does not result in the formation of DPNH, and (*b*) DPNH does not appear when the irradiation is carried out in the presence of dissolved hydrogen, a method which has been used by Stein & Weiss (1949) to increase the concentration of free hydrogen atoms in irradiated solutions. It is therefore apparent that this alternative explanation is not correct. DPNH can be formed only when ethanol is present in the absence of air,

and it is likely that the agent responsible for producing it is the C_2H_4OH radical. There is insufficient evidence available to be able to write equations explaining the production of DPNH by this radical, but in any case the alcohol dehydrogenases cannot work by this particular reaction because they are not inhibited by dissolved oxygen.

Theorell & Bonnichsen (1951) have shown that liver alcohol dehydrogenase (ADH) forms a complex with DPN, both in the oxidized and in the reduced form. This complex reacts with ethanol as follows (Theorell & Chance, 1951):

$$\text{ADH}-\text{DPN}^+ + C_2H_5OH \rightleftharpoons \text{ADH}-\text{DPNH} + H^+ + CH_3CHO. \quad (5)$$

There is much recent evidence that enzymic oxidations and reductions are free-radical reactions (George, 1952; Parravano, 1951), and as there are many points of resemblance between the enzymic and the γ-ray reductions of DPN, it seems likely that reaction (5) might be found to take place by a free-radical mechanism. Work is in progress to obtain further information on the radiation chemistry of this system.

SUMMARY

1. Gamma radiation acts on aqueous ethanol to produce acetaldehyde. The effect of dose and ethanol concentration on the yield is described for air-saturated solutions.

2. The yield of acetaldehyde in oxygen-free solutions is smaller than in the presence of air, and when DPN^+ is added the yield increases and DPNH appears. DPN^+ solutions do not produce DPNH when irradiated alone in air, or in the absence of oxygen, or in the presence of hydrogen.

3. When DPN^+ is added to air-saturated ethanol solutions the acetaldehyde yield is almost the same as in the absence of DPN^+ and DPNH does not appear.

4. It is considered that the free C_2H_4OH radical is responsible for the production of DPNH. This reaction is inhibited by dissolved oxygen and does not occur as a step in the action of the alcohol dehydrogenases.

The author wishes to thank Prof. P. B. Moon, F.R.S., for his encouragement of this work, and Dr K. F. Chackett for helpful discussions.

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Pancreatic Trypsin Inhibitor

1. PREPARATION AND PROPERTIES

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Two specific trypsin inhibitors are known to be present in pancreas; one, soluble in 2·5 % trichloroacetic acid, is a dialysable polypeptide which was crystallized by Kunitz & Northrop (1936). The other inhibitor is non-dialysable, and precipitated by trichloroacetic acid; it was obtained as a heterogeneous product in only small amounts (Kazal, Spicer & Brahinsky, 1948). The polypeptide inhibitor of Kunitz & Northrop formed a crystalline compound with trypsin, devoid of either inhibitor or trypsin activity. Its molecular weight was reported to be about 6000, but no evidence for purity of the final product was presented, and the analytical figures were admitted to be of uncertain significance.

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The object of the present investigation was to prepare this polypeptide inhibitor in amounts sufficient to establish its homogeneity, and then to obtain data on its amino-acid composition and mode of combination with trypsin.

The low inhibitor content of pancreas (yielding only 1 mg./kg. of fresh pancreas) rendered impractical the preparation of the desired amounts of inhibitor by the method of Kunitz & Northrop, which starts with the residues of trypsin preparation from fresh pancreas. Industrial pancreas residues from which insulin had been extracted (kindly made available by British Drug Houses Ltd.), proved to be a suitable source of inhibitor. Deproteinized extracts of these residues were about one-fifth as active as the starting material used by Kunitz & Northrop, so considerable preliminary purification had to be effected. The steps used in this preliminary purification are described in the present paper, together with tests for homogeneity and analytical data on the inhibitor. The following paper (Green & Work, 1953) describes kinetic data on its combination with trypsin, and a further paper describes its ultraviolet-absorption spectrum (Beaven & Green, 1953). A preliminary report of the work has been already made (Green & Work, 1951).

METHODS

Estimation of protein concentration. Protein concentration was estimated by measuring, with a Beckman photoelectric spectrophotometer, the optical density, E, at 280 mμ., using a 1 cm. cell. Trypsin was determined in 0·0025 N-HCl (pH 3), using the factor 0·585 to convert E to mg. trypsin/ml. (Kunitz, 1947). Inhibitor was measured in 0·01 M-phosphate buffer at pH 7, the factor 0·83 being used to convert E to mg. inhibitor/ml.; this factor was determined on three salt-free lyophilized inhibitor preparations dried at 80° *in vacuo*. During purification of inhibitor, E_{280} was checked at intervals against N content.

Estimation of trypsin activity. This was done by a modified version of the haemoglobin digestion method of Anson (1938), the amounts of material, time and temperature of reaction being reduced to conserve inhibitor and to avoid non-specific inhibition by split products.

Trypsin solution. Trypsin was prepared by the method of McDonald & Kunitz (1946), recrystallized twice and dried at −10°. The stock trypsin solution contained about 1 mg. crystalline trypsin/ml. of 0·0025 N-HCl and was stable for about 1 week at 3°. The exact concentration of trypsin was calculated from the value of E_{280}, and the solution was diluted approx. 1 in 10 with water immediately before use to give a solution containing about 4 μg. protein/ml. ($E_{280}=0·075$); the dilute solution lost 2–4% of its activity in 30 min. at 25°.

Substrate solution. Dried crystalline carboxyhaemoglobin (3·6 g.), prepared by the method of Jope (1948), was dissolved in 116 ml. of a 33% (w/v) aqueous solution of urea (recrystallized from 95% ethanol). N-NaOH (12·0 ml.) was added with stirring and the solution was left at 25° for 30–60 min. The pH was adjusted to 7·5 with 18·5 ml. of a solution made from urea (40 g.) and M-KH_2PO_4 (100 ml.). This solution, containing 3·2 mg. protein N/ml., was stable for several weeks at 3°.

Digestion. This was carried out at 25° for 5 min. in 10 ml. conical flasks (up to 20 in number); volumes were measured with semi-automatic pipettes which worked on the principle of the micropipettes of Linderström-Lang & Holter (1931). Trypsin solution (44 μg./ml.) was measured into the flasks and the volume made up to 1 ml. with water; after a few minutes at 25°, substrate solution (1 ml.), also at 25°, was added to successive flasks at regular time intervals (15 sec. or more), and after exactly 5 min., each digestion was stopped by 5% (w/v) trichloroacetic acid (2 ml.). The contents of each flask were centrifuged after 15 min. and the E_{280} of the supernatant liquid was measured, using as a blank the supernatant fluid from one digestion tube which was prepared by mixing trichloroacetic acid with trypsin before the substrate was added (Kunitz, 1947). E for the blank was checked against water and was usually between 0·16 and 0·21; if it was higher, fresh substrate solution was made.

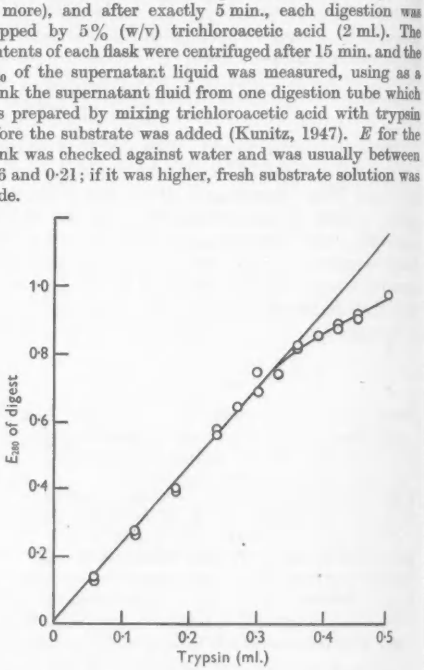


Fig. 1. Calibration curve for determination of trypsin units (TU) from E_{280} of digest; the two quantities are identical below $E_{280}=0·78$. Concentration of trypsin solution 44 μg./ml.

Trypsin unit. One trypsin unit (TU) was defined as the amount of trypsin giving digestion products with $E_{280}=1·0$ under the conditions of estimation. A standard trypsin calibration curve (Fig. 1), plotting E against trypsin added, was linear (i.e. TU $=E$) up to about $E=0·8$. Above this value, a correction for non-linearity was made from the curve, which was constructed afresh for each new specimen of trypsin used. From the curve, 1 TU was contained in 0·43 ml. of trypsin solution, equivalent to 19 μg. of trypsin protein, i.e. 1 TU/ml. = 19 μg./ml.

Estimation of inhibitor activity. The unit of inhibitor activity (IU) was defined as the amount of inhibitor which inactivated 1 unit of trypsin at pH 7. Specific activity, used to express inhibitor strengths, was defined as inhibitor units/ml. of solution of unit optical density at 280 mμ. and pH 7 (IU/ml., $E=1$).

For estimation of activity, the inhibitor, dissolved in 0·01 M-phosphate buffer pH 7, was measured into the digestion flasks and the volume was made up to 0·5 ml. with buffer. Trypsin (0·5 ml. to give final trypsin concentration 1·0 TU/ml.) was added and, after 5 min. standing at 25° to allow combination with inhibitor to take place, the subsequent procedure followed that used for trypsin estimation, duplicate inhibitor-free controls being run in every case. The blank was not affected by the inhibitor unless very impure samples were tested; in such cases a correction was applied afterwards, calculated from the E_{280} of the original inhibitor solution and its final dilution in the supernatant digest. At least three different amounts of inhibitor solution were added to a constant amount of trypsin. The slope of the straight line resulting from plotting volume of inhibitor added against trypsin activity remaining (from trypsin standard curve if necessary) represented the value IU/ml. (Fig. 3, —·—).

Electrodialysis. In the preliminary experiments a four-compartment cell (Work, 1951) with cellophan (no. 400) membranes was used; subsequently, the apparatus was changed to that depicted in Fig. 2. The solution to be dialysed was placed in a 2 l. jar containing the electrodes; the cathode (2) was a silver tube, through which cooling water was passed, and the anodes (3) were two platinum spirals wound on glass tubes and connected through Hg contacts at the bottom of each tube. The electrodes were each enclosed in a cellophan sac (5). Permeability to inhibitor of different samples of cellophan tubing varied considerably and a suitable batch was found only by trial and error. Outside the cathode sac was an annular glass ring (4) which could be moved by hand up and down the cellophan surface to remove any precipitated material; outside this (not shown) was a glass cooling coil. The main compartment was stirred mechanically. Mains voltage (220 V. d.c.) was applied and the current kept below 1·6 amp. by means of a variable resistance, in order to maintain the temperature of the cathode compartment below 30°. The entire contents of anode and cathode compartments were changed whenever the pH of the catholyte rose above 11.

Precipitation curves. These were constructed by varying either the amount or the pH of the precipitant added to constant amounts of inhibitor; the filtrates (suitably diluted) were examined for both protein content (E_{280}) and inhibitor activity, corrections being made for the effects of the precipitants. The curves were analysed by the specific property solubility test of Falconer & Taylor (1946, 1947). Precipitants were added to inhibitor solutions contained in Emich microfiltration vessels (porosity 3) which were then shaken gently for 14 hr. at 37°; subsequent filtration through the sintered-glass disk was carried out under a positive pressure of N_2.

Precipitants. (*a*) Buffered NaCl. Weighed amounts of NaCl (A.R.) were added to inhibitor dissolved in 5 ml. of McIlvains citrate-phosphate buffer (0·067 M). Since addition of NaCl lowered the pH of the buffer, the requisite pH for each NaCl concentration had to be predetermined. (*b*) 3M-Phosphate buffer, pH 6·5. Different amounts of stock buffer (prepared by half neutralization of KH_2PO_4 (A.R.) with KOH (A.R.), Butler & Montgomery, 1933) were added to aqueous inhibitor solutions.

Analytical methods. Salt-free inhibitor was obtained by dialysis at 2° against distilled water, using collodion membranes, made by Dr H. Gutfreund, which were impermeable to inhibitor. The salt-free material was freeze-dried and finally dried at 80° *in vacuo* before determining E_{280}/mg./ml. Amide N was estimated after 2 hr. hydrolysis at 100° in 6 N-HCl, the NH_3 produced after alkalization being distilled in a Conway cell into 0·001 N-HCl. Total N was estimated by Dumas or by Conway distillation.

Amino-acid analyses. These were mostly carried out on hydrolysates obtained by 24 hr. boiling with 6 N-HCl, using two-dimensional paper chromatography, first solvent phenol/water (NH_3 atmosphere), second solvent collidine/lutidine/water (Dent, 1948). Size and strength of each ninhydrin spot at several intensities near the minimum visible level were compared by eye with spots of similar intensities produced with measured amounts of each amino-acid (Lindan & Work, 1951), 50–500 μg. of inhibitor being used. Methionine was estimated as methionine sulphone after molybdate-catalysed peroxide oxidation (Dent, 1948). Cystine was examined as cysteic acid on one-dimensional phenol chromatograms run on oxidized hydrolysate, but estimation proved difficult owing to variable destruction by H_2O_2; cystine was therefore estimated by electrometric titration of unhydrolysed inhibitor as described later. Leucine and isoleucine were separated by *tert.*-amyl alcohol/water with diethylamine atmosphere (Work, 1949); tyrosine and histidine, separated by the butanol/acetic acid mixture described by Campbell, Work & Mellanby (1951), were developed with Pauly reagent (Dent, 1948), 750 μg. of hydrolysed inhibitor being used to show the absence of histidine. Tryptophan and tyrosine were estimated spectro-

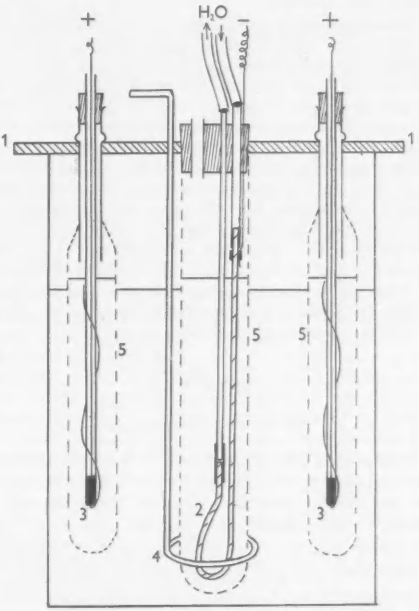


Fig. 2. Electrodialysis apparatus. 1, hard-board support; 2, cathode; 3, anodes; 4, annular stirrer; 5, cellophan sacs.

photometrically (Goodwin & Morton, 1946) on unhydrolysed inhibitor (see Beaven & Green, 1953).

Cystine estimation. Unhydrolysed inhibitor was dissolved (0·5 mg./ml.) in 0·3 N-H_2SO_4 containing 8 M-urea. This solution was reduced at a stirred Hg cathode (described by Dohan & Woodward, 1939) contained in a 30 ml. beaker; 0·5 ml. samples were taken at intervals and the liberated SH groups were titrated amperometrically against 0·01 N-$AgNO_3$ by the method of Kolthoff & Harris (1946), the $AgNO_3$ being standardized against cystine reduced by the same method. The presence of urea accelerated reduction (Fraenkel-Conrat, Mohammad, Ducay & Mecham, 1951) and, although it rendered the electrode sluggish, it had no effect on the end-point.

End-group and lysine assay. Inhibitor (15 mg.) was treated with fluorodinitrobenzene according to Sanger (1949) and the derivative hydrolysed with 6 N-HCl at 105° for 8 hr. The ethereal extract of the hydrolysate was examined for amino-acid dinitrophenyl (DNP) derivatives by paper chromatography (Biserte & Osteaux, 1950). DNP-lysine and DNP-arginine in samples of the aqueous extract were estimated spectrophotometrically (Sanger, 1945) after separation on silica-gel columns using the solvent system ethyl methyl ketone:ether (66:34 by vol.) equilibrated with 2 M-Na_2HPO_4 (Blackburn, 1949). Corrections were applied for losses on the column and for breakdown of the DNP-lysine and arginine by carrying out control hydrolyses.

Filter-paper electrophoresis. This was carried out on 150 μg. of inhibitor in the apparatus described by Durrum (1950) using buffers of pH 8–10.

RESULTS

Shape of inhibition curve

When partially purified inhibitor was being assayed, a straight-line relationship existed between amount of inhibitor added and trypsin units remaining. With crystalline inhibitor, a departure from linearity was observed at low inhibitor concentrations with certain trypsin preparations (Fig. 3). The magnitude of the effect, although dependent on the particular sample of trypsin used, was not directly connected with the trypsin activity of the sample, nor was it changed by recrystallization. It was also unaffected by change in trypsin and substrate concentrations. The trypsin sample (called standard trypsin) finally chosen for estimation purposes was one which gave imperceptible falling off (—·—).

Production of inhibitor from crude pancreas residues

Preliminary experiment on purification. Damp acid-ethanol-containing pancreas residues from insulin preparation were extracted with 0·25 N-sulphuric acid and the extract 80 % saturated with ammonium sulphate. The precipitate dissolved in water had no proteolytic activity, but after removal of protein with hot trichloroacetic acid and saturation with magnesium sulphate at pH 3, a precipitate was obtained which had inhibitory activity corresponding to an inhibitor content of 1–2 %. Subsequent experiments showed that preliminary heating of the damp pancreas residues to 70° to remove ethanol before extraction doubled the inhibitor activity of the magnesium sulphate precipitate. This product, containing about 50 % magnesium sulphate and 5 % nitrogen, was the starting material for subsequent preparations, but could not be directly purified by the method of Kunitz & Northrop (crystallization of inhibitor-trypsin compound) since the impurities rendered crystallization impossible.

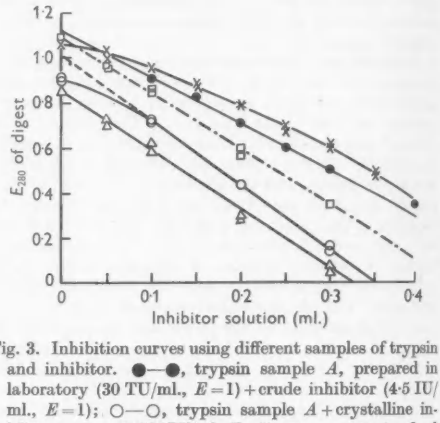


Fig. 3. Inhibition curves using different samples of trypsin and inhibitor. ●—●, trypsin sample *A*, prepared in laboratory (30 TU/ml., $E=1$) + crude inhibitor (4·5 IU/ml., $E=1$); ○—○, trypsin sample *A* + crystalline inhibitor prepn. (i) (220 IU/ml., $E=1$); □—·—□, standard trypsin, prepared in laboratory (30 TU/ml., $E=1$) + crystalline inhibitor prepn. (i); △—△, commercial trypsin (20 TU/ml., $E=1$) + crystalline inhibitor prepn. (i); ×—×, commercial trypsin (30 TU/ml., $E=1$) + crystalline inhibitor prepn. (i); – – – –, indicates degree of departure from linearity.

Salt fractionation. Optimal conditions for salt fractionation were determined by constructing salting-out curves and pH-solubility curves using sodium chloride as precipitant. Fig. 4 shows the effect of pH on the solubility of crude inhibitor in saturated sodium chloride solution, both total protein concentration (E_{280}) and inhibitor activity of filtrate being plotted against pH. At pH 6, the precipitate contained 28 % of the total peptide but no inhibitor; at pH 3 all the inhibitor was precipitated, but only 82 % of total peptide. Fig. 5 shows the effect of increasing salt concentration at pH 2·5. There is a single break in the curve; the less soluble component was without inhibitor activity; the more soluble component, although impure as shown by its low activity, behaved as a single phase (log *E* plotted against ionic strength gave a straight line). Either type of fractionation was suitable as a

method of purification; the former was chosen, being slightly more convenient, the fraction precipitated in saturated sodium chloride solution between pH 5·8 and pH 3 being retained.

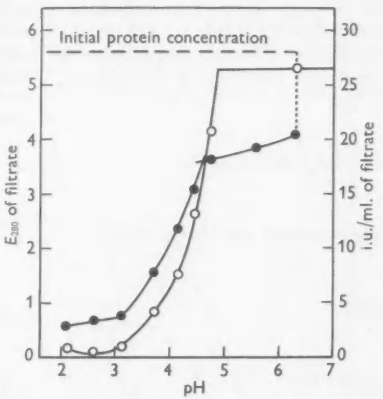


Fig. 4. Effect of pH on solubility of crude inhibitor in saturated NaCl solution. ●—●, total protein concentration in filtrate measured by E_{280}; ○—○, inhibitor units in filtrate.

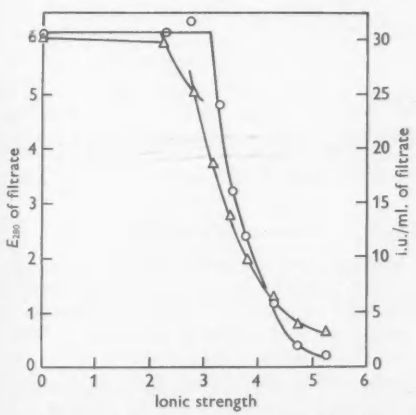


Fig. 5. Solubility of crude inhibitor in NaCl solution at pH 2·5. △—△, total protein concentration in filtrate (E_{280}); ○—○, inhibitor units in filtrate.

Electrodialysis. Salt-precipitated inhibitor was substantially freed from sodium chloride by dialysis through cellophan for a short period during which practically no inhibitor was lost. The remainder of the sodium chloride was removed during the first stages of electrodialysis, when little peptide moved from the centre compartment. During the next few hours, about 5% of the total peptide, containing 60–70% of the inhibitor, migrated to the cathode but negligible amounts went to the anode. The pH of the centre compartment had little effect on inhibitor mobility; even at pH 12 little peptide material with negligible inhibitor activity went to the anode. At pH as low as 1, little further peptide moved to the cathode, beyond that which moved at neutral reactions. Repeated electrodialysis of the catholyte effected no further purification. In all experiments, about 20% of the original inhibitor activity remained in the centre compartment, and was presumably due to some other substance which inhibits trypsin.

Electrodialysis was adopted as a preparative step after salt fractionation, and following these processes the compound of the inhibitor with trypsin could be crystallized.

Large-scale inhibitor preparation

The scheme (see p. 262) for the preparation of inhibitor from pancreas residues is given and experimental details of a typical preparation follow. All weights refer to damp filter cakes; activities are expressed as IU/ml., $E = 1$.

Extraction of crude inhibitor. Oven-dried pancreas residue (100 kg.) from insulin extraction was suspended in 0·25 N-H_2SO_4 (140 l.) for 24 hr. with occasional stirring. The liquid, separated by centrifugation, was 80% saturated with $(NH_4)_2SO_4$, and the centrifuged precipitate (1·3 kg.) was dissolved as far as possible in water (3·2 l.). After straining to remove lumps, the solution was heated to 60° in batches (1·5 l.) and mixed with an equal volume of boiling trichloroacetic acid (50% w/v). After standing for 4 min. at 80°, the mixture was cooled rapidly to 30° by pouring through cooling coils, then centrifuged; the supernatant was adjusted to pH 3 and saturated with $MgSO_4$. The precipitate, containing inhibitor, was collected in a Sharples centrifuge (250 g., activity 10). Any solid found floating on the centrifugate was combined with the main bulk.

(Since the activities of different batches of pancreas were variable, it was found advisable to run pilot extractions, with a few kg. of pancreas residues, as far as this stage.)

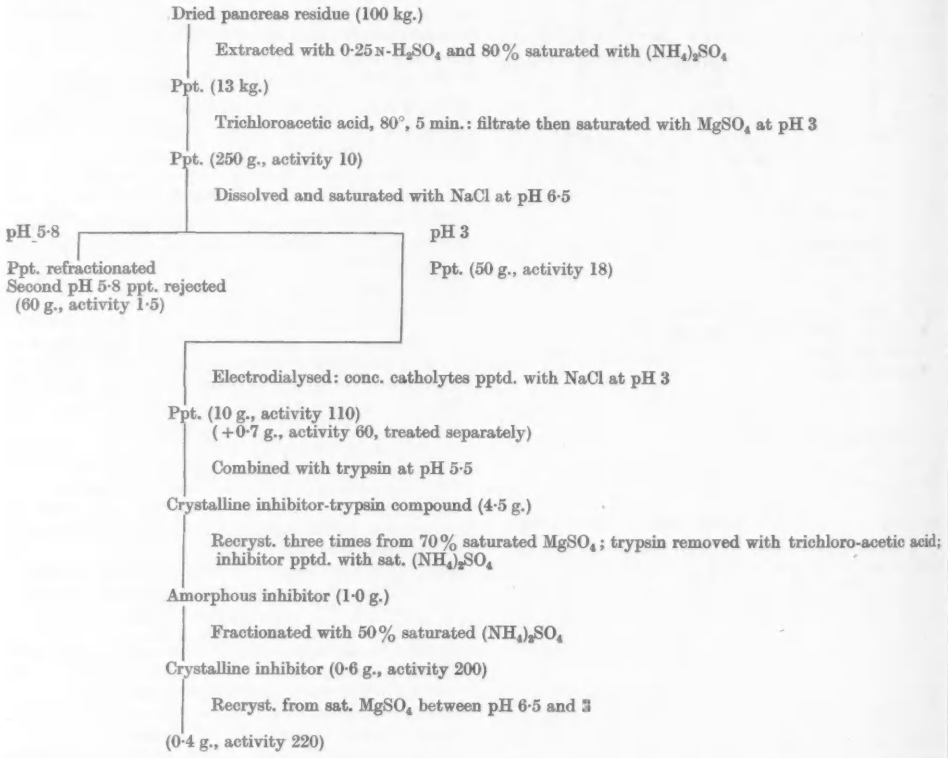
Salt fractionation. The crude inhibitor (250 g.) was dissolved in water (5 l.) and the pH adjusted to 6·5. NaCl (1·7 kg.) was added and the pH was adjusted to 5·8; after 3 hr., the precipitate was removed in the Sharples centrifuge; the supernatant liquid was acidified to pH 3, when a yellow gummy precipitate was formed, containing most of the inhibitor. The pH 5·8 precipitate was redissolved in water and refractionated as above. The combined pH 3 precipitates (50 g., activity 18) were dissolved in a minimum of water (approx. 50 ml.) at 40°, and partly freed from NaCl by dialysis in a rotating cellophan sac against 1 l. of distilled water (at 40°, to prevent the solution from setting to a gel). The water was changed at hourly intervals until the dialysate no longer tasted salty.

Electrodialysis. The inhibitor solution was diluted to 1·2 l. and electrodialysed in two portions as described in Methods. The various catholyte fractions were combined into two batches according to their activities, and concentrated tenfold *in vacuo*; inhibitor was precipitated by saturating the

solution with NaCl at pH 3. (Yield 10 g., activity 110 + 0·7 g., activity 60).

Subsequent steps in producing crystalline inhibitor followed the method of Kunitz & Northrop (1936) except that the inhibitor-trypsin compound was recrystallized from 0·7 sat. $MgSO_4$, not from sat. $MgSO_4$ (see scheme).

Recrystallization of inhibitor. Crystalline inhibitor (0·6 g.) was dissolved in 0·1 M-acetate buffer pH 6·5 (6 ml.) and the solution saturated with $MgSO_4$. A little partially crystalline material precipitated and was filtered off. The filtrate was acidified to pH 3; a precipitate which formed immediately crystallized rapidly and was filtered off after standing overnight. (Yield: 0·3–0·4 g. after two recrystallizations.) The fraction precipitated at pH 6·5 was recrystallized separately and yielded a slightly less active material.


Dried pancreas residue (100 kg.)
Extracted with 0·25 N-H_2SO_4 and 80% saturated with $(NH_4)_2SO_4$
Ppt. (13 kg.)
Trichloroacetic acid, 80°, 5 min.: filtrate then saturated with $MgSO_4$ at pH 3
Ppt. (250 g., activity 10)
Dissolved and saturated with NaCl at pH 6·5
pH 5·8
Ppt. refractionated
Second pH 5·8 ppt. rejected
(60 g., activity 1·5)
pH 3
Ppt. (50 g., activity 18)
Electrodialysed: conc. catholytes pptd. with NaCl at pH 3
Ppt. (10 g., activity 110)
(+0·7 g., activity 60, treated separately)
Combined with trypsin at pH 5·5
Crystalline inhibitor-trypsin compound (4·5 g.)
Recryst. three times from 70% saturated $MgSO_4$; trypsin removed with trichloro-acetic acid; inhibitor pptd. with sat. $(NH_4)_2SO_4$
Amorphous inhibitor (1·0 g.)
Fractionated with 50% saturated $(NH_4)_2SO_4$
Crystalline inhibitor (0·6 g., activity 200)
Recryst. from sat. $MgSO_4$ between pH 6·5 and 3
(0·4 g., activity 220)

Activity of inhibitor preparations

Three main batches (i, ii and iii) of inhibitor were prepared, final specific activities of crystalline materials being 220, 130 and 120, respectively. A small amount prepared from fresh pancreas (Kunitz & Northrop, 1936) had activity 180. Removal of salt by dialysis caused a fall in activity of preparation (i) to 160 units, while the activity of preparation (iii) rose to 140 units, a small amount of peptide material being detected in the early dialysates of preparation (iii).

Homogeneity of inhibitor

Precipitation curves with increasing concentrations of phosphate buffer, pH 6·5, were constructed for crystalline inhibitor preparation (ii) (activity 130) and, for purposes of comparison, a sample (activity 70) purified only as far as electrodialysis. Plotting inhibitor activity or total peptide concentrations against ionic strength of buffer gave smooth curves (Fig. 6) for crystalline inhibitor which were straight lines when plotted semi-logarithmically (Fig. 7). The impure material gave broken lines (Fig. 6, – – –) suggesting the presence of about four components, none of which was closely associated with inhibitor activity. Application of the specific property solubility test (Falconer & Taylor, 1946, 1947) showed that precipitation of inhibitor was directly proportional to precipitation of peptide for the crystalline inhibitor (Fig. 8), indicating that the inhibitor was behaving as a

homogeneous substance under the conditions of test.

Filter-paper electrophoresis. When tested by filter-paper electrophoresis using buffers in the pH range 8–10, crystalline inhibitor (ii) appeared to be homogeneous, migrating at all pH's to the cathode as a single sharp spot. The impure specimen gave a long streak. The magnitude of the electro-endosmotic effect prevented a determination of the isoelectric point, but the inhibitor proved to be at least as basic as lysine.

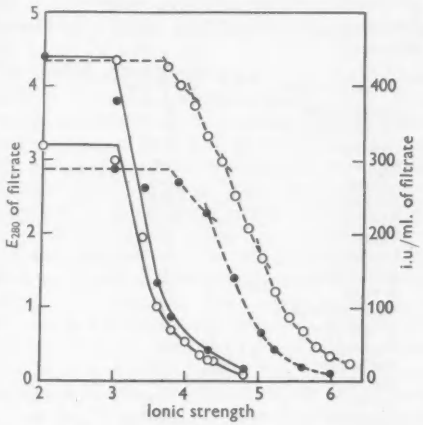


Fig. 6. Salting-out curves of two inhibitor samples in potassium phosphate buffer, pH 6·5. ○, total protein concentration in filtrate (E_{280}); ●, inhibitor units in filtrate. ——, Crystalline inhibitor (prepn. (ii), activity 130 IU). – – –, Electrodialysed inhibitor (activity 65 IU).

Properties of inhibitor

General. The crystalline salt-free inhibitor was a white solid, soluble in water, 70 % (w/v) methanol and ethanol, and 50 % (v/v) acetone. It showed a solubility minimum at pH 3, both in strong inorganic salt solutions and in aqueous solution; it could in fact be recrystallized by changing the pH of a concentrated aqueous solution from 5·5 to 3. Its solubility in organic solvents was low in relation to its water solubility, as shown by distribution experiments in butanol/acetic acid and in chloroform/methanol/water, and by its failure to move on paper chromatograms with the following solvent systems: collidine/lutidine/water; *n*-butanol/N-ammonia; *n*-butanol/20 % acetic acid; *sec.*-butanol/3 % acetic acid; tetrahydrofurfuryl alcohol/water (80:20). In phenol/water (acetic acid atmosphere) it ran with the solvent front.

The molecular weight of preparation (ii), determined by Dr H. Gutfreund by osmotic pressure measurements, was 9000 ± 500. Values of E_{280}/mg./ml. for preparations (i), (ii), and (iii) were 0·84, 0·82 and 0·837, respectively.

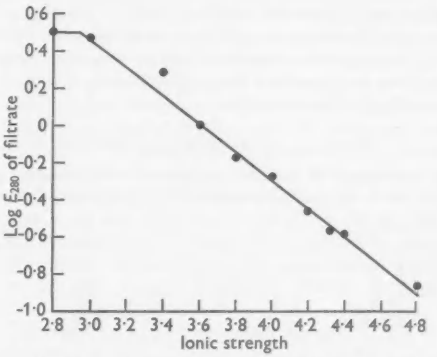


Fig. 7. Salting-out curve of crystalline inhibitor prepn. (ii) in potassium phosphate buffer, pH 6·5, expressed semi-logarithmically.

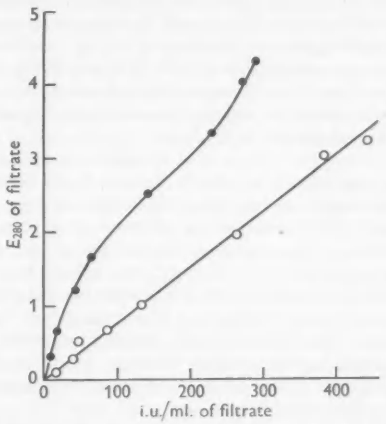


Fig. 8. Relation between total protein and inhibitor activity left in solution with increase in ionic strength of potassium phosphate buffer, pH 6·5. ○—○, Crystalline inhibitor (prepn. (ii), activity 130 IU); ●—●, electrodialysed inhibitor (activity 65 IU).

The inhibitor was stable in organic solvents and in dilute acids at 100°, but was slowly inactivated in alkaline solutions at room temperature (Table 1). It was precipitated by sodium picrate, but some activity was lost on regeneration of the free base.

Chemical composition. The mean analytical results on the three samples are as follows: C, 47·6; H, 6·7; N, 16·5; amide N, 0·75 %. The results were identical except in the case of nitrogen which was 16·0, 17·0 and 16·5 % for preparations (i), (ii) and

(iii), respectively. The sulphur content was not determined because of shortage of material, but from the results of the determination of amino-acid content, given below, would be about 2·5%. The Fiske and Subbarow test for phosphate, carried out on the hydrolysed inhibitor, was negative. The inhibitor gave a negative Molisch test for carbohydrate and positive ninhydrin, biuret, Sakaguchi and Pauly diazo reactions.

Table 1. *Alkali stability of inhibitor*

(Percentage of inhibitor activity left after standing at 25° under the conditions shown. 0·14% (w/v) solution.)

Time (hr.) ...	1	2	5	24	72
pH 9·3	—	—	—	84	46
pH 10·0	—	—	—	79	61
pH 10·8	—	—	—	53	—
pH 11·9	—	—	82	49	—
0·1 N-NaOH	—	—	—	29	—
N-NaOH	66	27	—	—	—

Amino-acid composition. All amino-acid analyses were carried out in parallel on preparations (i) and (ii), but no difference between the two samples was found, except that the first hydrolysate prepared from preparation (ii) showed on chromatograms a molybdate/peroxide-stable spot in the position of methionine sulphoxide (Green & Work, 1951); this was not found in subsequent hydrolysates from the same material, so it was presumed to be a peptide which had resisted hydrolysis.

Electrolytic reduction of inhibitor in acid solution, carried out to reduce cystine S—S linkages before amperometric titration of sulphydryl groups, caused 80% decrease in activity after 30 min. reduction. (Reduction of cystine under the same conditions was not complete even after 2 hr., but addition of urea completed the reduction.) A steady titration value, indicating the presence of 7·2% cystine, was obtained with inhibitor after 45 min. reduction in the presence of urea. Unreduced inhibitor gave a zero titration, showing the absence of free sulphydryl groups.

Table 2 shows the results of all amino-acid analyses. An amino-acid mixture resembling the hydrolysed inhibitor gave paper chromatograms similar to those from hydrolysed inhibitor.

End-group analysis and lysine content. Preparations (i) and (ii) were treated in parallel and no significant difference was found between them. The ethereal extract from the hydrolysate of the DNP-inhibitor was only a very pale yellow; paper chromatograms gave two spots, with R_F values different from those of any known DNP-amino-acid but identical with those of dinitroaniline and dinitrophenol, presumably decomposition products from hydrolysis of basic DNP derivatives. The end group, if any, was therefore a basic amino-acid and, since there was no histidine in the inhibitor, was probably arginine. Samples of the aqueous layer were applied to ethyl methyl ketone : ether columns; in front of the DNP-lysine band was a faint band. This was proved to be DNP-arginine by its R_F value on the column relative to lysine, by paper chromatography, and by the value of E_{350}/E_{390}. Contamination of the arginine band by colourless *o*-DNP-tyrosine (indicated by absorption at 300 mμ.) prevented an estimation of DNP-arginine from E_{350}/E_{390}.

Table 2. *Approximate amino-acid composition of salt-free lyophilized inhibitor, estimated* (±15%) *by paper chromatography, except where stated otherwise*

Amino-acid	g./100 g. of inhibitor	Residues/mol. of inhibitor
Aspartic acid	8	6
Glutamic acid	8	6
Cystine*	7·2	3
Serine	3·5	3
Glycine	7·5	12
Threonine	4·5	4
Alanine	13	18
Valine	3	2
Leucine	3	2
Isoleucine	1	1
Proline	6	5
Phenylalanine	8	5
Tyrosine†	9	5
Tryptophan†	0	0
Methionine	1·6	1
Histidine	0	0
Lysine‡	7·3	5
Arginine	15	9
Amide N	0·74	5
Total N	16·5	
End group (arginine)	—	1

* Estimated amperometrically.
† Estimated spectrophotometrically (Beaven & Green, 1953).
‡ Estimated through DNP derivative.

When further columns were run, a third faint band was always observed just in front of DNP-arginine; its strength relative to the arginine band was 0·4:0·6. This band was possibly due to an arginine-containing peptide which resisted acid hydrolysis, since the products of 1 hr. alkaline hydrolysis (Mills, 1950) gave spots on chromatograms similar to those given by control hydrolysates of DNP-arginine, except for the presence of an additional unidentified purple spot. The quantitative values showed that this band plus the arginine band added up to one end group per molecule, but this cannot be taken as an accurate assay.

DISCUSSION

Activity measurements. Measurements of inhibitor activity must be carried out under conditions which are completely reproducible and where non-specific inhibition of trypsin by proteolysis products

is at a minimum. The use of synthetic substrates should achieve this, but with benzoylarginine amide as substrate the digestion rate was so slow as to be inconvenient for routine testing. As digestion rates with denatured protein substrates are faster, the substrate chosen was carboxyhaemoglobin, crystallized to ensure reproducibility, trypsin activity being measured by a slight modification of Anson's (1938) method. The reaction velocity during the 5 min. digestion period was constant to within 10%, thus the digestion values were approximate measures of initial reaction velocities. Duplicate estimations of trypsin activity were repeatable to within 2%, while duplicate inhibition curves agreed within 5%.

The departure from linearity of the inhibitor curve at low inhibitor concentrations with certain trypsin samples might be explained by the presence in the trypsin of some impurity which reacted with the inhibitor. A likely contaminant is denatured trypsin (Bier & Nord, 1951) but experiments described in a following paper (Green & Work, 1953) showed that heat- or alkali-denatured trypsin does not combine with inhibitor to any significant extent. Considerable evidence is accumulating which suggests that trypsin is not homogeneous, either enzymically (Werle, Kehl & Koebke, 1950; Pope & Stevens, 1951) or electrophoretically (Anderson & Alberty, 1948). Green (unpublished) has found that the activity of trypsin can be raised by passing it through a Celite partition column (Martin & Porter, 1951).

Properties of inhibitor. The three different preparations of inhibitor had identical elementary analyses and optical properties, and the two samples examined had similar amino-acid compositions; but inhibitory activities were slightly different. The activities were not altered by recrystallization, even after change of pH and of precipitating salt, but in two cases they were affected by the final dialysis carried out in order to remove salts. The origin of these differences appeared to be in the original pancreas residues, which showed great and inexplicable variations of inhibitory activity; the most active starting material yielded the most active crystalline inhibitor, but many batches of pancreas residues showed negligible inhibitor activity.

Homogeneity of the least active sample was investigated by precipitation curves, and the specific property solubility test indicated that the inhibitor behaved as a single entity under the conditions of test. The variation of activity must be attributed to some small variation in structure undetectable in this large molecule by the analytical methods employed, or to a difference in arrangement of amino-acids. The inhibitor as prepared may consist of a family of peptides of slightly differing biological activity but with chemical properties so similar as to prevent separation by any of the methods used (Synge, 1949).

Amino-acid estimations were mainly carried out by paper chromatography and were consequently only approximate, but they enabled a rough picture of the amino-acid composition to be obtained. All the amino-acids commonly occurring in proteins were present with the exception of tryptophan (Beaven & Green, 1953) and of histidine; the methionine content was only one residue/mole. Amperometric titration showed the absence of sulphydryl groups, but after electrolytic reduction there were six sulphydryl residues/mole showing that the inhibitor contained three cystine residues. This figure differs from the preliminary figure (one residue) quoted previously (Green & Work, 1951) which was determined by rough paper chromatography. Subsequently more careful chromatography with peroxide-treated controls gave the same value as that obtained electrometrically. Arginine was found to constitute the sole end group; an accurate end-group assay was not obtained, but it was possible to say that there was not more than one end group per molecule. The high arginine content (about nine residues per mole) accounts for the basicity of the molecule as a whole; five of the twelve acidic amino-acid residues were present as amides and another five acidic charges would be neutralized by the free amino groups of the five lysine residues.

The basic nature of the inhibitor, originally revealed by electrodialysis, was unexpected, since Kunitz crystallized the inhibitor at pH 5·5. In this respect, the inhibitor resembles lysozyme, a basic protein of mol.wt. 15000 which crystallizes at pH 4 (Alderton & Fevold, 1946). The exact isoelectric point of the inhibitor has not been determined, but it is certainly higher than pH 10. The analytical results on the inhibitor differ from those given by Kunitz & Northrop (1936) whose N value (11·25%) is unusually low for a peptide; the molecular weight of 9000 found for our preparation is also different from the value of 6000 found by Kunitz & Northrop.

Comparison with other trypsin inhibitors. In addition to the pancreatic inhibitor, specific inhibitors for trypsin have been identified in soya bean, lima bean, egg white, colostrum and *Ascaris* (Kunitz, 1947; Fraenkel-Conrat, Bean & Lineweaver, 1949; Fraenkel-Conrat, Bean, Ducay & Olcott, 1952; Schmitz, 1939; Laskowski & Laskowski, 1951; Collier, 1941); they are all stable proteins, with the exception of ovomucoid inhibitor which contains 25% carbohydrate. Only pancreatic, soya bean and colostrum inhibitors have been obtained crystalline, but preparations of ovomucoid and lima bean inhibitors each gave a single boundary

on electrophoresis. Molecular weights of soya bean and ovomucoid inhibitors are 24000 and 28000 and one pancreatic inhibitor is also of relatively high molecular weight; the other inhibitors have molecular weights of 10000 or less. The isoelectric points of soya, lima and ovomucoid inhibitors lie between 4 and 5.

Complete amino-acid analysis has been carried out only on lima bean inhibitor; it bears no resemblance to pancreatic inhibitor except for the absence of tryptophan, also noted in colostrum inhibitor.

SUMMARY

1. Crystalline pancreatic trypsin inhibitor was obtained from industrial pancreas residues remaining after insulin extraction. Three separate preparations yielded materials of similar composition but of slightly differing activities.

2. The homogeneity of one preparation was investigated by the specific property solubility test.

3. The properties of the inhibitor were those of a basic peptide; it migrated during electrodialysis through cellophan to the cathode when the pH of the centre was below 10.

4. Elementary analysis was C, 47·6; H, 6·7; N, 16·5%.

5. The molecular weight was 9000 ± 500.

6. The amino-acid composition was determined mostly by paper chromatography. Histidine was absent and there was only one methionine residue/mol. of inhibitor. Cystine was determined amperometrically on unhydrolysed inhibitor after electrolytic reduction in the presence of urea.

7. End-group assay showed arginine to be the terminal amino-acid.

8. The inhibitor was slowly inactivated in alkaline solution at room temperature, but was stable in hot dilute acid.

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The Chemical Nature of the Second Hydrogen Peroxide Compound Formed by Cytochrome *c* Peroxidase and Horseradish Peroxidase

1. TITRATION WITH REDUCING AGENTS

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The haemoprotein enzyme peroxidase, containing iron as ferriprotoporphyrin, catalyses very effectively the oxidation of fairly complicated organic molecules like phenols and amines by hydrogen peroxide. This action is intimately connected with its ability to form intermediate compounds with hydrogen peroxide in the absence of the reducing agent, for such compounds react very rapidly with the reducing agent if it is then added. Three such compounds recognizable by their colour and absorption spectrum can be formed depending on the experimental conditions, and they are related as follows:

$$\underset{\substack{1\text{ mole}\\(\text{brown})}}{\text{Peroxidase} + H_2O_2} \rightarrow \underset{\substack{1\text{ mole}\\(\text{green})}}{\text{complex I}} \xrightarrow[\text{transition}]{\text{spontaneous}} \underset{(\text{pale red})}{\text{complex II}}$$

$$\underset{(\text{pale red})}{\text{complex II}} \xrightarrow{\text{excess } H_2O_2} \underset{(\text{deep red})}{\text{complex III}}$$

Following the successful application of the Michaelis-Menten enzyme-substrate complex theory to other enzymic reactions, these compounds have been regarded as enzyme-substrate complexes, peroxidase-H_2O_2 or peroxidase-OOH^- and named accordingly complexes I, II and III. The second and third compounds were first described by Keilin & Mann (1937) who showed that both could be reduced back to the ferric form of the enzyme. Theorell (1941) showed that complex II was not formed immediately when hydrogen peroxide was added to peroxidase but that a transient green-coloured compound, complex I, was formed first which very rapidly changed into complex II. Chance (1949*a*, 1951*b*, *c*) has carried out an extensive kinetic investigation of peroxidase action and interpreted the results in terms of the following Michaelis-Menten mechanism:

$$\text{Per.OH} + \text{HOOR} \rightleftharpoons \text{Per.OOR (I)} + H_2O, \quad (1)$$

$$\text{Per.OOR (I)} \rightarrow \text{Per.OOR (II)}, \quad (2)$$

$$\text{Per.OOR (II)} \xrightarrow{\text{spontaneous decay}} \text{Per.OH}, \quad (3)$$

$$\text{Per.OOR (II)} + AH_2 \rightarrow \text{Per.OH} + \text{ROH} + A. \quad (4)$$

This has been widely accepted and accounts also for the comparable catalytic activities of complexes formed with hydrogen peroxide and alkyl hydroperoxides of the type ROOH. The symbols Per.OH represent the ferric form of the enzyme with an OH group bound to the iron atom, and Per.OOR (I) and Per.OOR (II) the two enzyme-substrate complexes. The mechanism is a modification of that originally suggested by Michaelis & Menten in that the products are formed by the complex reacting with another molecule, AH_2, and not undergoing a unimolecular decomposition.

Such a mechanism has the following disadvantages:

(i) It gives no clue to differences in structure to account for the widely diverse spectroscopic and magnetic properties of the complexes. (ii) It is difficult to reconcile the type of chemical reaction shown in step (4), where four covalent bonds are broken and reformed, with the high speed of the reaction. (iii) It is difficult to envisage reaction (4) proceeding with a single electron transfer reducing agent, like ferrocytochrome *c*, without involving a two-stage reaction and consequent elaboration of the mechanism (see Chance, 1951*a*). (iv) It is difficult to understand the transition from complex I to complex II, in reaction (2), particularly in view of Chance's finding that the transition is speeded up by all reducing agents that react with complex II (1949*b*).

A mechanism avoiding all these disadvantages was suggested by George (1952*a*) which is based on the discovery by George & Irvine (1951, 1952) that the compound formed from metmyoglobin and hydrogen peroxide does not contain H_2O_2 or O_2H^- as a component part of its structure because the reduction of this compound back to metmyoglobin involves only one equivalent. The compound thus contains iron with an effective oxidation number of +4. If complex II of peroxidase is also of this type the transition from complex I to complex II is easily explained as a single equivalent reduction process and the paradox vanishes in understanding how a strong oxidizing agent, Per.OOR (I), changes into another strong oxidizing agent, Per.OOR (II),

Table 1. *Calculation of the equilibrium constant for the formation of the CcP-cyanide complex on the assumption that the CcP sample was lower in concentration than the value of 30 μM determined from d_{410}.*

Cyanide present (μM)	Observed percentage complex formed	Equilibrium constant × 10^{-5}, [CcP] in μM				
		30	28·0	27·5	27·0	20·0
14·1	45·7	12·1	5·30	4·68	4·25	1·01
20·8	64·0	8·2	5·10	4·66	4·35	2·10
27·5	78·0	6·8	5·30	4·98	4·95	2·78

in the presence of a reducing agent without chemical reaction occurring.

As yet the chemical structures of complexes I–III are unknown, and for this reason they are referred to in this paper as compounds I–III because the term complex implies that the structure contains H_2O_2 or O_2H^-. In the biochemical literature the oxidizable substrates are often termed hydrogen donors or electron donors: since this also suggests a preferred reaction mechanism, which still remains to be established, the more general term reducing agent is adopted here.

This paper presents evidence that compound II of horseradish peroxidase and cytochrome *c* peroxidase contain the iron in the quadrivalent state or an equivalent structure. A preliminary account of the experiments has already appeared (George, 1952*a*, *b*).

EXPERIMENTAL

Symbols. Horseradish peroxidase and cytochrome *c* peroxidase have been abbreviated to HRP and CcP.

E_{425} and ΔE_{425} represent the optical density of a solution at 425 mμ. and the optical density increment observed when a reaction has taken place. Measurements at other wavelengths are indicated by the appropriate subscript.

In some chemical mechanisms the symbol Fe_p^{3+} has been used to denote the ferriprotoporphyrin iron atom in the enzyme. Its net charge within the co-ordination unit is really +1 due to replacement of two pyrrolic H atoms, but the use of Fe_p^{3+} facilitates comparison with ionic iron reactions and makes valency changes simpler to follow.

Methods. The reactions were carried out at room temperature, 28–32°, in 1 cm. cuvettes of a Beckman Model DU spectrophotometer and optical densities measured in the usual way. A.R. grade reagents were employed. Phosphate buffers of pH 5·3–5·4 and pH 7·0 were made by mixing the appropriate solutions of 0·067 M-KH_2PO_4 and 0·067 M-$Na_2HPO_4.2H_2O$ and diluting to give a total phosphate concentration of 0·01 M. Merck's Perhydrol was diluted to give H_2O_2 stock solutions of about 50 μM; the concentration was determined spectrophotometrically at 230 mμ. where mε = 0·0724.

A several-times recrystallized sample of HRP was used which had been prepared by Theorell's (1942) method; concentrations were calculated on the basis mε at 402 mμ. = 81 appropriate to this type of preparation. The ratio E_{402}/E_{280} was 2·7 indicating about 90% purity (Theorell & Maehly, 1950). Cytochrome *c* had been prepared by Keilin & Hartree's (1945) method.

Four different samples of CcP were used which were prepared by the method of Abrams, Altschul & Hogness (1940) except that the treatment with alumina gel was omitted. From their data the ratio E_{410}/E_{270} for a pure sample can be estimated as 1·47 (Altschul, Abrams & Hogness, 1942). On this basis the present enzyme samples, 1, 2, 3 and 4 were only 23, 23, 11 and 14·5% pure. The contribution of the protein diluent to the optical density in the Soret region was obtained using sample 2 in the following experiment. A concentrated cyanide solution was added to the enzyme solution in 0·01 M-phosphate buffer, pH 7·0, which was nominally 30 μM with respect to CcP, as determined from E_{408}, taking mε = 93, according to the earlier workers. The cyanide complex has a broad absorption band at 544 mμ. in the visible region and the percentage of complex formed was obtained by averaging optical density increments at 535, 540 and 545 mμ., which agreed to within 1%. In Table 1 equilibrium constants are calculated from these data on the assumption that the enzyme concentration had the values 30·0, 28·0, 27·5, 27·0 and 20·0 μM. It can be seen that a value 27·5–28·0 μM gives the most concordant figure for the equilibrium constant, which shows that the enzyme concentration based simply on E_{410} is about 8% too high. This factor has been used in calculating the true enzyme concentration for all four samples, since the variation in the diluent protein concentration would only change this at most by 1% which is within experimental error. Enzyme activity tests were carried out for all four samples by observing the catalysis of the oxidation of ferrocytochrome *c* with hydrogen peroxide: this gave values between 5 and 10 times greater than that obtained by the earlier workers: this can only be attributed to idiosyncrasies in the test itself.

The absorption spectra in the Soret region of CcP and a number of its complexes which have been used in the present investigation are given in Figs. 1 and 2. The spectra of CcP and compound II are from mean values obtained using all four enzyme samples. For the other compounds sample 1 was used. At all wavelengths a contribution to the optical density by the diluent protein amounting to 8% of E_{410} has been assumed and the resulting optical densities adjusted according to E_{408} for 1·0 mM-CcP = 93 cm. The character of these absorption spectra, particularly the symmetry of the band of the ferro-CcP-CO complex, is a good indication that no other haemoproteins were present.

Experimental conditions for the study of compound II of HRP and CcP

The spontaneous decomposition of compound II is faster the higher the absolute concentration and so, to make the necessary corrections for this as low as possible, enzyme concentrations of 1–2 μM were usually used. Such solutions have optical densities of the order 0·080–0·160 in the Soret region, with optical density increments accompanying a chemical reaction of the order 0·060, permitting measurements with an accuracy of about 2%. The spon-

taneous decomposition is presumably the reaction of compound II with reducing matter present in the enzyme preparation either as trace impurities or actual reducing groups on the enzyme molecule itself. There was a considerable difference between the samples of HRP and CcP in this respect. Compound II of CcP was found to be the more stable with a half-life of the order of 10–30 min. at 30° for a 1–3 μM solution. Compound II of HRP had a half-life of about 3–5 min. at the start of the investigation when the enzyme sample had been prepared for 3 months. Later, however, when the sample had been prepared for 6–9 months, the half-life had increased and the compound was as stable as that from CcP. Except with the first experiments with HRP, corrections for this spontaneous decomposition were negligible with both enzymes in the low concentration experiments.

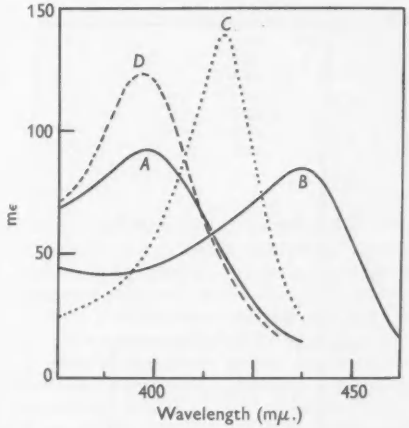

Fig. 1. Spectra of CcP and some of its derivatives. *A*, CcP; *B*, ferro-CcP; *C*, ferro-CcP-CO; *D*, CcP-fluoride.

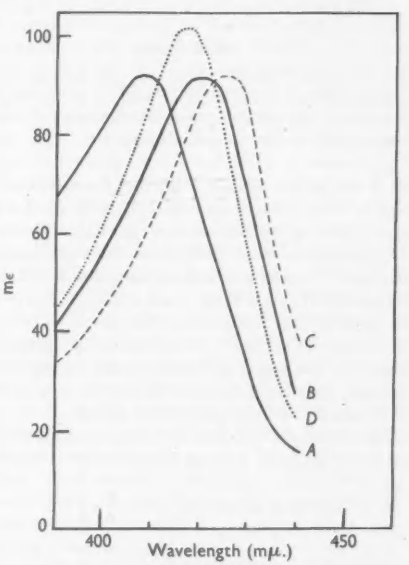

Fig. 2. Spectra of CcP, compound II, and its cyanide and azide complexes (*A*, *B*, *C* and *D* respectively).

Determination of the absorption spectrum of compound II

There is another stage in the peroxidase-H_2O_2 reaction where this differing reducing capacity has an effect and it again appeared that the HRP contained less reducing matter than the CcP. When H_2O_2 was added to CcP the Soret band, which has its maximum at 408 mμ. for the free enzyme, shifted toward the red with the maximum now at 420 mμ. shown in Fig. 2. Using 1 μM concentration this reaction was found to be complete by the time the first spectrophotometric measurement was made some 15 sec. after mixing. If about a fivefold molar excess of H_2O_2 was present and small amounts of a reducing agent like *p*-cresol added of the order of the enzyme concentration, no alteration was observed in the height or position of the Soret band of compound II. This was taken to mean that the transition from compound I to compound II was already complete since reducing agents speed the transition, and that the enzyme preparation itself contained enough reducing matter to effect the rapid transition. Further addition of *p*-cresol in excess of the H_2O_2 added reduced compound II back to the free enzyme, which was completely recovered within experimental error.

With the HRP under similar conditions the absorption band on adding peroxide had its maximum at about 420 mμ. but was less intense: the addition of small amounts of reducing agents caused the band to increase in intensity so that finally it had the same extinction coefficient as the free enzyme (Fig. 3). Evidently insufficient reducing matter was present in the enzyme sample to complete the transition from compound I to compound II. This change in spectrum is in accord with Chance's (1949*c*) data for the absorption spectrum of compound I, which has an absorption band of low intensity at 410 mμ., since a mixture of compounds I and II would give the type of spectrum shown in Fig. 3*A*. As in the case of CcP, reducing agents in excess were found to regenerate the enzyme quantitatively. The spectrum obtained by adding a small amount of *p*-cresol to HRP with a sixfold excess of H_2O_2 present has been taken to represent the full conversion of HRP into compound II, and concentrations of compound II have been obtained by optical density measurements at 420, 425, 430 and 435 mμ. taking as standard $\Delta\mu\varepsilon_{425} = 0{\cdot}044$ for its reduction back to the free enzyme.

These results with CcP and HRP, showing that the band of compound II is of about the same intensity as that of the free enzyme, suggest that the spectrum reported by Altschul *et al.* (1942) is low by about 4% by reason of incomplete conversion from compound I to compound II which might well occur with a more pure preparation than the one used here. On this assumption $\Delta\mu\varepsilon_{425}$ for full conversion is 0·0365. The values obtained with the present enzyme samples are somewhat higher: sample 1, 0·043; sample 2, 0·0415 and 0·048; sample 3, 0·0395 and sample 4, 0·0385.

A mean value of 0·0405 has been used in subsequent calculations. The reason for the discrepancy is unknown, but it is reasonable that $\Delta\epsilon_{425}$ for CcP should be less than that for HRP because the absorption bands of CcP and its compound II are closer together as a comparison of Figs. 2 and 3 shows.

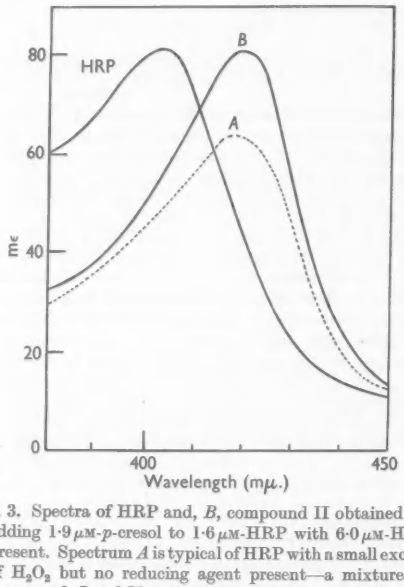

Fig. 3. Spectra of HRP and, *B*, compound II obtained by adding 1·9 μM-*p*-cresol to 1·6 μM-HRP with 6·0 μM-H_2O_2 present. Spectrum *A* is typical of HRP with a small excess of H_2O_2 but no reducing agent present—a mixture of compounds I and II (1·0 μM-HRP and 1–3 μM-H_2O_2).

Titration of compound II of CcP and HRP with potassium ferrocyanide

Preliminary experiments showed that the reaction of compound II with ferrocyanide ions resembles that of the metmyoglobin-H_2O_2 compound, for the reaction is faster in more acid solution (George & Irvine, 1952). At pH 7·0 the reaction had a half-time of about 15 min. with 1·4 μM-CcP compound II and 0·4 μM-$Fe(CN)_6^{4-}$, which is too slow to permit an accurate titration since the spontaneous decomposition of compound II had a half-time of about 30 min. under these conditions. At pH 5·4, however, the reaction was much faster (Fig. 4). The velocity constant for the reaction can be computed as about 10^6 M^{-1} sec.$^{-1}$

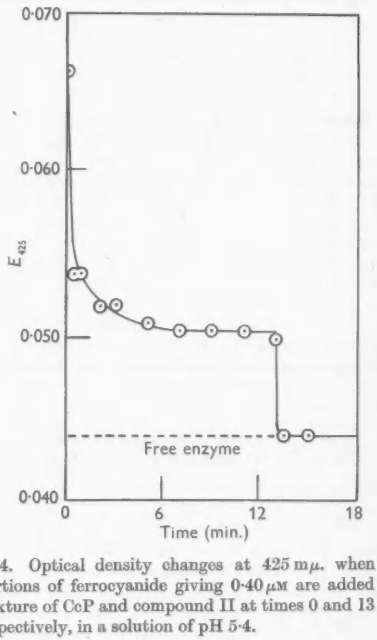

Fig. 4. Optical density changes at 425 mμ. when two portions of ferrocyanide giving 0·40 μM are added to a mixture of CcP and compound II at times 0 and 13 min. respectively, in a solution of pH 5·4.

and a rough comparison of the data shows the reaction rate to vary according to at least the first power of the hydrogen-ion concentration. The curve in Fig. 4 shows that an optical density change of 0·016 cm.$^{-1}$ at 425 mμ. accompanies the reaction of compound II with 0·04 μM-$Fe(CN)_6^{4-}$, i.e. 0·016/(0·40 × 0·0405) = 1 mole of compound II reacts with 1 mole $Fe(CN)_6^{4-}$. The results of similar experiments are given in Table 2 and all show that the reduction of compound II to the free enzyme involves only one oxidizing equivalent.

A satisfactory titration of compound II of HRP was more difficult, for, as shown above, complete

Table 2. *Titration of CcP compound II with ferrocyanide at* pH 5·4, *obtained by optical density measurements at* 425 mμ.

Ferrocyanide concentration (μM)	ΔE_{425}	Compound II reacting (μM)	Compound II / Ferrocyanide
0·15	0·0065	0·160	1·07
0·24	0·009	0·22	0·92
0·40	0·016	0·395	1·00
0·56	0·020	0·495	0·88
0·75	0·030	0·74	0·99
1·00	0·0365	0·90	0·90
		Mean	0·96 ± 0·60

Table 3. *Titration of HRP compound II with ferrocyanide at* pH 5·4, *obtained by optical density measurements at* 425 mμ.

Ferrocyanide concentration (μM)	ΔE_{425}	Compound II reacting (μM)	Compound II / Ferrocyanide
0·15	0·006	0·14	0·93
0·30	0·015	0·34	1·13
0·50	0·022	0·50	1·00
0·70	0·027	0·62	0·89
0·75	0·028	0·64	0·85
0·90	0·031	0·71	0·79
0·90	0·035	0·79	0·88
		Mean	0·92 ± 0·08

conversion of the enzyme into compound II is only effected by adding small amounts of reducing agents with excess H_2O_2 present. These conditions are clearly incompatible with a titration. In the experiments below, the full amount of compound II was allowed to form, using a H_2O_2 concentration insufficient to give the maximum optical density change to ensure that no excess H_2O_2 was left; ferrocyanide was then added, and the titration result obtained interpreted on the basis that a little compound I was present.

Compound II of HRP was found to react more rapidly with ferrocyanide than CcP compound II, and the optical density of the solution after mixing was constant after about 40 sec. Table 3 sets out the results of several titrations calculated on the basis that only free HRP and compound II were present, showing that 0·92 mole of compound II reacts with 1 mole of ferrocyanide. An upper estimate of the amount of compound I actually present can be obtained from the observation that with 1 μM-HRP, $E_{425} = 0{\cdot}029$ and the optical density after adding excess H_2O_2 to give the maximum change was 0·066. For 1 μM compound I and compound II, $E_{425} = 0{\cdot}025$ and 0·073, hence about 14% of the HRP was present as compound I under these conditions. In the titration experiments where less H_2O_2 was used, a smaller fraction of compound I will have been present but it cannot be determined from these data; yet, whatever it might have been, it will raise the stoicheiometric factor of the reaction of compound II with ferrocyanide. Calculation shows that some 6% of compound I present could raise the stoicheiometric factor by about 0·09 and so it is justifiable to conclude that the factor is really unity.

It was observed that a freshly made solution of ferrocyanide did not react as rapidly as one that had stood for a few hours. The probable explanation of this is the formation of a small amount of $Fe(CN)_5H_2O^{3-}$ in the solution which reacts faster with compound II, permitting a catalysis of the ferrocyanide-compound II reaction through the subsequent reaction of $Fe(CN)_5H_2O^{3-}$ with $Fe(CN)_6^{4-}$. There was no evidence of any irreversible change taking place in such ferrocyanide solutions that might have altered the reducing capacity and so invalidate the titration results. This is borne out by similar results obtained using other reducing agents.

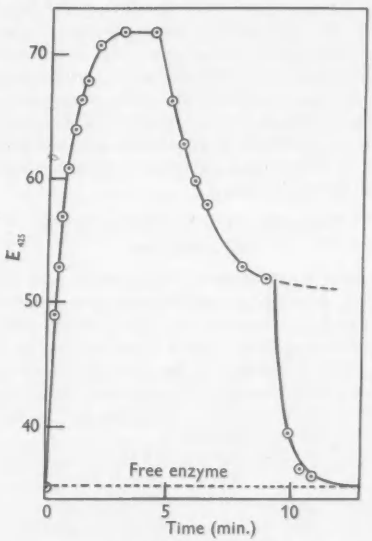

Fig. 5. Optical density changes at 425 mμ. when two portions of ferrous ion giving 0·5 and 1·0 μM are added to a mixture of HRP and compound II at times 4·3 and 9·3 min. respectively, in a solution of pH 5·4.

Titration of HRP compound II with ferrous ion

Spectrophotometric examination showed that a fivefold excess of Fe^{2+} reduced compound II quantitatively to the free enzyme at pH 5·4. The reaction is, however, slower than with ferrocyanide as shown in Fig. 5, from which the velocity constant can be estimated as about 10^4 M^{-1} sec.$^{-1}$. An interesting feature appearing in Fig. 5 is the relatively slow formation of compound II obtained after the enzyme sample was about 6 months old. The concomitant stability of the compound once

Table 4. *Titration of HRP compound II with ferrous ions at* pH 5·4, *obtained by optical density measurements at* 425 mμ.

Ferrous ion concentration (μM)	ΔE_{425}	Compound II reacting (μM)	Compound II / Ferrous ion
0·5	0·021	0·48	0·96
0·7	0·025	0·57	0·82
1·0	0·035	0·80	0·80
		Mean	0·86 ± 0·07

formed was shown by the optical density remaining constant for at least 2 min. This enabled titration with Fe^{2+} to be carried out, for the reduction took about 5 min. under these conditions. From Fig. 5 it appears that an optical density change of 0·021 at 425 mμ. occurs when compound II reacts with 0·05 μM-Fe^{2+}, i.e. 0·48/0·5 = 0·96 mole compound II reacts with 1 mole Fe^{2+}. The results of two other titrations are included in Table 4 giving a mean stoicheiometric factor of 0·86. Correction for the amount of compound I present would raise this figure and, since the slow formation of compound II indicates less active reducing matter in the system, it is likely that more compound I was present here than in the ferrocyanide ion titration. It is thus justifiable to regard the two titrations as being in substantial agreement with a stoicheiometric factor of unity.

Titration of CcP compound II with ferrocytochrome c

In the titrations with ferrocyanide and ferrous ion the reasonable assumption was made that compound II, present in excess, reacted with the full amount added to give ferricyanide and ferric ion. Ferrocytochrome *c* has the added advantage that its concentration changes can be measured spectrophotometrically, affording an additional check on the stoicheiometry.

In the following experiment H_2O_2 was added to a 1·50 μM solution of CcP giving 1·34 μM concentrations of compound II (see note later on concentrations), 0·05 ml. of a concentrated cytochrome *c* solution, in which 71 % of the pigment was in the reduced state, was then added so that the resulting solution was 1·58 μM in cytochrome *c*. Optical densities were measured at 413 and 433 mμ., the isosbestic points for CcP-compound II and ferriferrocytochrome *c* respectively, before and after the addition of cytochrome *c*. The reaction between compound II and ferrocytochrome *c* was observed to be very rapid, being complete by the time the first optical density was measured 15 sec. after the addition. The stoicheiometry of the reaction was calculated as follows.

Table 5 gives the optical density changes observed. At 433 mμ. the optical density of μM-cytochrome *c* was 0·0285 and the increment for conversion of CcP into compound II 0·0295: thus the contribution of cytochrome *c* to the optical density of the final solution 0·101 was 1·58 × 0·0285 = 0·045. So the CcP in this solution contributed (0·101–0·045) = 0·056. The optical density change accompanying the reaction of compound II with ferrocytochrome *c* was therefore (0·0875–0·056) = 0·0315, corresponding to 0·0315/0·0295 = 1·07 μM-compound II.

Table 5. *Data for the titration of CcP compound II with ferrocytochrome* c

Solution	Optical densities 410 mμ.	413 mμ.	433 mμ.
1·50 μM-CcP alone	0·140	0·131	0·048
With 2 μM-H_2O_2 present	—	0·131	0·0875
With 1·58 μM-cytochrome *c* then added (71 % reduced)	—	0·292	0·101
The same cytochrome *c* solution alone	—	0·190	0·045

At 413mμ. the contribution of CcP and compound II to the optical density was 0·131 which agrees well with the estimated value of 0·132 obtained from the concentration 1·50 μM and a previously determined value of $\mu\varepsilon$ 0·088. The cytochrome *c* in the final solution thus had an optical density at 413 mμ. of 0·292–0·132 = 0·160. If no oxidation had occurred the optical density would have been 0·190, hence a change of 0·030 accompanied the oxidation by compound II.

The optical density increment for the oxidation of ferrocytochrome *c* to ferricytochrome *c* at 413 mμ. was found to be $\Delta\mu\varepsilon$ = 0·288, hence this change corresponds to the formation of 0·030/0·0280 = 1·05 μM ferricytochrome *c*. The original concentration of ferrocytochrome *c* in the sample was 0·71 × 1·58 μM = 1·12 μM and, since an excess of compound II was added, this full amount was available for oxidation. Thus these results show that

1·07 μM-compound II + 1·12 μM-ferrocytochrome *c* → 1·07 μM-CcP + 1·05 μM-ferricytochrome *c*.

It is clear that the stoicheiometric factor is unity and the deviation attributable to experimental error. In the calculation the observed optical densities at 410 and 413 mμ. have been assumed to be entirely due to CcP, neglecting the fact that about 8 % is due to the protein diluent because this does not affect the stages of the calculation when the total optical density is involved. The appropriate value of the optical density increment at 433 mμ. for the conversion of CcP to compound II has been obtained from the spectra on the basis that the value at 425 mμ. is 0·0405 as has been used in the previous titrations.

An attempt to bracket the oxidation-reduction potential for the reduction of compound II to the free enzyme

In a survey of the reaction of the secondary compounds with a variety of reducing agents it was found that both were reduced by chloroiridite ions with complete regeneration of the free enzyme as in ferrocyanide or ferrous ion reduction, although a larger amount was required in the case of CcP compound II. No reduction was observed with ferrous tris-2:2′-dipyridyl or tris-*o*-phenanthroline ions, but in the case of HRP where some compound I was present with compound II an increase in the intensity of the absorption band was observed like that shown in Fig. 3 which occurred when *p*-cresol was added, suggesting that these ferrous complexes can reduce compound I to compound II.

DISCUSSION

The nature of compound II

The titration of compound II with the various reducing agents all showed that it is reduced to ferriperoxidase Fe_p^{3+} in a one equivalent reduction:

$$\text{compound II} + e^+ \rightarrow Fe_p^{3+}. \quad \text{(reaction } a\text{)}$$

The compound thus has only one oxidizing equivalent in a reaction regenerating the free enzyme compared with the two oxidizing equivalents originally present in H_2O_2. This result requires that H_2O_2 or its anion O_2H^- cannot be a component part of the structure of compound II and so it should no longer be regarded as an enzyme-substrate complex (Chance, 1943, 1949*a–c*, 1951*b*, *c*). An iron compound whose oxidation-reduction reactions are expressed by reaction (*a*) formally contains iron with an oxidation number of +4. Several possibilities may be considered for the structure of such a compound: (i) simple quadrivalent iron Fe^{4+} bound in the porphyrin ring either covalently, or ionically as Fe^{2+}, and Fe^{3+} are bound in ferro- and ferri-haemoproteins; (ii) a derivative of quadrivalent iron with, for instance, O^- bound as in the structure FeO^{3+}, which in the case of ionic iron would be the ferryl ion and correspond to the vanadyl ion VO^{2+}; (iii) a biradical structure in which tervalent iron forms 'one end' of the radical, the other end being a normal radical grouping at a methine carbon atom, a pyrrolic carbon atom or some other atom within the conjugated network of porphyrin ring and haemoprotein linkage; (iv) a higher oxidation state of the haematin group in which the electron has been removed from a π-orbital common to the ring as a whole. The existence of this type of oxidation state has recently been demonstrated by Cahill & Taube (1951) for the structurally similar metal phthalocyanines containing copper, iron, cobalt, zinc or aluminium in tetrasulphonated derivatives. Structures (i) and (iv) arise by simple electron transfer and only differ as to which is the lower lying energy level, whereas structures (ii) and (iii) require bond-breaking reactions both in the formation and subsequent reaction of the compound. The magnetic susceptibility of compound II formed from H_2O_2 has not been measured, but that from CH_3OOH, which has an identical spectrum and similar titration curve (George, 1953) and may therefore be regarded as the same compound, has $\chi_m = 3500 \times 10^{-6}$ c.g.s. units (Theorell, Ehrenburg & Chance, 1953). A quadrivalent iron compound in which the bonds are essentially covalent requires two unpaired electrons per iron atom and this value for χ_m is a little greater than the theoretical value of 3390×10^{-6} c.g.s. units calculated for two unpaired electrons on a 'spin only' basis. The susceptibility value is therefore in accord with the oxidation-reduction behaviour of the compound as represented in reaction *a*, although Theorell, Ehrenburg & Chance choose to regard it as a ferric complex analogous to ferriperoxidase cyanide which has a susceptibility $\chi_m = 2970 \times 10^{-6}$ c.g.s. units.* The susceptibility value does not help to decide between the four structures for compound II given above except to rule out a purely ionic type of binding. The following chemical evidence does, however, suggest that bond-breaking reactions, as contrasted to simple electron transfer, are involved in the reactions of compound II.

First the reduction by ferrocyanide ion, or $Fe(CN)_5H_2O^{3-}$, is faster the higher the hydrogen-ion concentration, as shown above. Secondly, the reaction of compound II with ionizable reducing agents like quinol, guaiacol and pyrogallol is independent of pH in the range 3·6–6·7 (Chance, 1949*a*) suggesting that the conjugate acid of these reducing agents is the reacting species. Thirdly, the autoxidation of these same reducing agents is faster in more alkaline solution which is usually attributed to a preferred electron transfer mechanism involving an anionic species. The contrast between these two systems and the hydrogen-ion dependency of the ferrocyanide reaction both favour the inclusion of hydrogen in the reduction of compound II. This can most simply be illustrated with a 'ferryl ion' structure:

$$Fe_pO^{2+} + H^+ + e^- \rightarrow Fe_p^{3+} + OH^-,$$
$$Fe_pO^{2+} + AH_2 \rightarrow Fe_p^{3+} + OH^- + AH,$$

but similar reactions can be written for the 'biradical' structure.

* Theorell, Ehrenburg & Chance (1952) more recently report a revised value of $\chi_m = 4850 \times 10^{-6}$ c.g.s. units for compound II formed from CH_3OOH. They no longer propose that it is a simple ferric complex like the cyanide complex, but discuss its susceptibility in terms of the ferryl ion structure suggested by George & Irvine (1951).

A tentative value of about $-1{\cdot}0$ V. (U.S.A. convention) or the compound II/ferriperoxidase couple is afforded by the observation that compound II is reduced by chloroiridite ions but not by ferrous tris-2:2′-dipyridyl or tris-*o*-phenanthroline ions which have oxidation-reduction potentials of $-1{\cdot}02$, $-1{\cdot}06$ and $-1{\cdot}14$ V. respectively (Hume & Kolthoff, 1943; Walden, Hammett & Chapman, 1933; Woo, 1931). The uncertainty arises because little is known of the chloroiridate-chloroiridate system in other than acid solution and because a difference in charge type in the case of the ferrous complexes might result in a decrease in reaction rate obscuring the fact that a reaction can occur. This latter factor is probably absent, however, since there is evidence that compound I does react with the ferrous complexes.

The formation of compound II

The suggestion was made recently that the transition from compound I to compound II is a reduction process (George, 1952*a*, *b*)

$$\text{compound I} + AH_2 \rightarrow \text{compound II} + AH. \qquad \text{(reaction } b\text{)}$$

Such a mechanism resolves the difficulty encountered with the enzyme-substrate complex theory in understanding how the transition is affected by trace-reducing matter in the enzyme samples and is speeded up by all reducing agents that react with compound II (Chance, 1949*b*).

According to this new mechanism compound II should be one oxidizing equivalent above the ferriperoxidase and this has now been established by the titrations with reducing agents. In addition, compound I should be two oxidizing equivalents above ferriperoxidase and so contain the original oxidizing capacity of the peroxide, as in fact has always been assumed in enzyme-substrate complex mechanisms. It is difficult to see how the two stages of the overall reaction could otherwise be accounted for in view of the oxidation-reduction properties of compound II; however, several of the experiments described above furnish evidence in support of this.

The spectra in Figs. 2 and 3 show that far less compound I is left in the final solution when peroxide is added to CcP as compared to HRP. The compound II titration data in Tables 2–5 show the stoicheiometric factor for the reduction of the CcP compound II to be nearly unity, whilst that for the HRP compound II is significantly lower. Reducing agents are known to speed the transition from compound I to compound II and so the only consistent explanation on the basis that both CcP and HRP secondary compounds have the same oxidation-reduction properties is that some reducing agent is used in the HRP experiments to convert compound I into compound II. This requires compound I to have a greater oxidizing capacity than compound II.

The experiments described in the next paper of this series (George, 1953) in which peroxide was added to a mixture of CcP and ferrocyanide show clearly that ferrocyanide is involved in oxidation-reduction competition reactions leading to the formation of compound II. More of the oxidizing capacity of the peroxide in the system can be accounted for under these conditions and the amount of ferrocyanide used in these preliminary reactions is approximately proportional to its initial concentration. This result would be expected according to reaction *b*. Following on these experiments Chance (1952) has shown that compound I can be reduced by ferrocyanide to compound II in a 1:1 reaction, by using a more acid solution where compound I is more stable, and employing a rapid-recording spectrophotometer.

The observation that the ferrous tris-2:2′-dipyridyl and tris-*o*-phenanthroline complexes give more compound II when added to a mixture of compound I and compound II without reducing compound II is further indirect evidence in favour. On the basis of reaction *b*, compound I but not compound II could have a high enough oxidation-reduction potential to oxidize the ferrous complexes.

A provisional value for the oxidation-reduction potential of the compound I–compound II couple may be obtained from the following reactions:

$$\text{peroxidase} + H_2O_2 \rightarrow \text{compound I},$$
$$\text{compound I} + H^+ + e^- \rightarrow \text{compound II} + H_2O,$$
$$\text{compound II} + H^+ + e^- \rightarrow \text{peroxidase} + H_2O,$$
$$H_2O_2 + 2H^+ + 2e^- \rightarrow 2H_2O,$$

where H^+ is included arbitrarily to balance the overall reaction. This has an oxidation-reduction potential $E^\circ = -1{\cdot}77$ V. which becomes $-1{\cdot}44$ V. at pH 5·3 which was used in the experiments. The equilibrium constant and hence the free-energy change accompanying the formation of compound I from H_2O_2 is not known with certainty (Chance, 1949*b*), but for methyl hydroperoxide an equilibrium constant of $3{\cdot}1 \times 10^5\ \text{M}^{-1}$ is given. This is a reasonable lower limit for the constant using hydrogen peroxide and calculation from the above equations on this assumption gives $-1{\cdot}6$ V. for the oxidation-reduction potential of the compound I/compound II couple. No upper estimate of the equilibrium constant can be made, but even if it were as high as $10^{10}\ \text{M}^{-1}$ the potential would only fall to $-1{\cdot}3$ V. These rough calculations based on the new mechanism thus indicate that compound I could be a more powerful oxidizing agent than compound II by about 0·3–0·6 V. in accord with the observed reduction of compound I but not compound II by the ferrous complexes.

The mechanism of peroxidase action when compound II is involved

Chance's assumption that compound II is an enzyme-substrate complex capable of reacting thus:

$$\text{Per.OOH} - \text{II} + \text{AH}_2 \rightarrow \text{Per.OH} + \text{H}_2\text{O} + \text{A}$$

was based on measurements with excess reducing agent present showing that the decomposition of compound II was first order and the observed velocity constant directly proportional to the concentration of reducing agent (Chance, 1949*a*, *d*). With conditions giving a first-order reaction of this kind the stoicheiometry is obscured and his data fit equally well the reactions

$$\text{compound II} + \text{AH}_2 \xrightarrow[\text{determining}]{\text{rate}} \text{peroxidase} + \text{AH}$$

followed by $2\text{AH} \rightarrow \text{A} + \text{AH}_2$,

or $\text{compound II} + \text{AH} \rightarrow \text{peroxidase} + \text{A}$,

resulting in an over-all reaction of the type

$$2\ \text{compound II} + \text{AH}_2 \rightarrow 2\ \text{peroxidase} + \text{A}$$

in conformity with the titration results reported in this paper. This new mechanism is of particular importance in the oxidation of ferrocytochrome *c* by the peroxidase system for it resolves the difficulty in formulating a reaction with compound II on a two equivalent basis (Chance, 1951*a*).

In view of the wide acceptance of compound II as an enzyme-substrate complex it is desirable to summarize its properties revealed by the above experiments which serve to distinguish it from a true complex of this kind. (1) The substrate, peroxide, is not a component of its structure. (2) It cannot dissociate to give the free enzyme and a valency-saturated product: it can only be reduced to the free enzyme.

Chance has shown that compound II can behave kinetically as a rate-limiting intermediate in peroxidase reactions, but it does not necessarily follow that a Michaelis-Menten mechanism suitably modified to allow for a bimolecular reaction between Per.OOH-II and AH_2 is operative. Rate-limiting intermediates are always found in catalysed oxidation-reduction reactions, although the initial state of the catalyst usually predominates in the stationary state.

In formulating a chemical mechanism for peroxidase action (as distinct from a complete kinetic mechanism) it is now essential to consider possible structures for compound I in relation to compound II, which is an intermediate that can now be recognized as belonging to the same class as a free radical or a semi-quinone, for it permits a stepwise mechanism with the transfer of 1 oxidizing equivalent at each step. In general, compound I may either still contain H_2O_2 or its anion O_2H^- in an ion-pair complex and fulfil the structural requirements of an enzyme-substrate complex, or it may possess the same oxidizing capacity as H_2O_2 in the form of a higher oxidation state of iron, in this case $+5$, or in some equivalent structure like Fe_pO^{3+}. The formation of compound II can be accounted for equally well in both mechanisms, as may be seen in the following reactions in which compound II is assumed to have the ferryl ion structure, Fe_pO^{2+}:

(i) compound I as an ion-pair complex:

$$Fe_p^{3+}O_2H^- + AH_2 \rightarrow Fe_pO^{2+} + AH + H_2O;$$

(ii) compound I as a higher valency state:

$$Fe_p^{3+} + H_2O_2 \rightarrow Fe_pO^{3+} + H_2O,$$

$$Fe_pO^{3+} + AH_2 \rightarrow Fe_pO^{2+} + AH + H^+,$$

(I) (II)

where Fe_p^{3+} represents the peroxidase iron atom.

It is not yet possible to decide to which class compound I belongs. There is no direct evidence that it is an enzyme-substrate complex capable of dissociating into free peroxidase and peroxide. On the contrary, George's (1952*b*) observations that peroxidases react with other oxidizing agents like HOCl, HOBr, BrO_3 periodate, ClO_2, ClO_3, ozone and persulphate in the presence of silver, but not cupric ions, favour the higher oxidation state theory. These experiments showed clearly that compound II was formed and the type of spectrum resembled that of a mixture with compound I as in Fig. 3*A*.

The formulation of a kinetic mechanism with reducing agents of the type AH_2 involves assumptions about the fate of the AH radicals. There is in addition the possibility that in the reactions for which data are available molecular oxygen was also a reactant. Even with ferrocytochrome *c* as the reducing agent unexpected side reactions occur. Chance (1950) found HRP and CcP were readily inactivated by the turnover of several separate additions of ferrocytochrome *c* and that with HRP the activity could be restored by the addition of a little *p*-phenylenediamine. These observations suggest that the reducing groups associated with the enzyme preparation play a dominant kinetic role, and until more is known of this behaviour no satisfactory kinetic mechanism can be advanced.

SUMMARY

1. Spectrophotometric titrations of compound II formed by interaction of peroxidase and hydrogen peroxide with reducing agents such as ferrocyanide, ferrocytochrome *c* and ferrous ions, show it to undergo a 1 equivalent reduction to ferriperoxidase, the initial oxidation state of the enzyme. Hydrogen peroxide or the anion O_2H^- cannot therefore be a component part of its structure and it should no longer be regarded as an enzyme-substrate complex,

Per.OOH, but as a compound in which the iron has an effective oxidation number of +4.

2. These results strongly support the following new mechanism for the interrelationship of compound I and compound II which necessitates a complete revision of the accepted mechanism for peroxidase action.

$$\text{Peroxidase} + H_2O_2 \rightarrow \underset{\text{(2 oxid. equiv.)}}{\text{compound I}} \xrightarrow[\text{reduction}]{\text{1 equiv.}} \underset{\text{(1 oxid. equiv.)}}{\text{compound II}}$$

$$\underset{\text{(1 oxid. equiv.)}}{\text{compound II}} \xrightarrow[\text{reduction}]{\text{1 equiv.}} \text{peroxidase}$$

3. Preliminary values of 1·3–1·6 and approx. 1·0 V. have been estimated for the oxidation-reduction potentials of the compound I/compound II and compound II/peroxidase couples respectively at pH 5·3 (European convention).

4. Four types of possible chemical structure for compound II containing iron with an effective oxidation number of +4 are discussed, together with two general structures available for compound I, either as a ferric complex or a derivative containing iron with an effective oxidation number of +5.

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Comparative Detoxication

1. THE METABOLISM OF SULPHADIMIDINE IN THE LOCUST

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Knowledge of the 'detoxication mechanisms' of higher animals is finding useful application in the design of more effective drugs (cf. Brodie, 1952; Williams, 1951), and it is possible that similar studies in insects may lead to more effective insecticides (cf. Brown, 1951). Very little is known, however, of the metabolic changes undergone by foreign organic compounds in the insect. Most of the information available deals with the dehydrohalogenation of halogenated compounds such as DDT (1:1:1-trichloro-2:2-di(*p*-chlorophenyl)ethane) (Perry & Hoskins, 1950; Ferguson & Kearns, 1949; Winteringham, Loveday & Harrison, 1951), but apart from this there are only a few isolated reports of metabolic changes in insects, for example, oxidation of salicin to salicylaldehyde and salicylic acid in

certain beetles (Hollande, 1909), the oxidation and probable conjugation of phenothiazine in the cockroach (Zukel, 1944) and the hydrolysis of pyrethrins in caterpillars (Swingle, 1934). No study has yet been made in insects of the conjugation mechanisms or oxidations which occur in animals (Williams, 1947); it is hoped to compare the known mechanisms of warm-blooded animals with those of the locust which is a large and easily obtained insect.

The conjugation reaction of the amino group was chosen for study since it can be easily followed by the Bratton-Marshall (1939) colorimetric method for aromatic amines. Furthermore, we wished to try different methods of administering compounds to locusts (cf. Shephard, 1939) and to find out how long it took the insect to excrete the drug. Sulphadimidine (Sulphamezathine, sulphadimethylpyrimidine, 2-(*p*-aminobenzenesulphonamido)-4:6-dimethylpyrimidine) was selected for the present study because, in the higher animals, it is readily absorbed from the gut, highly conjugated by acetylation and rapidly excreted (Krebs, Sykes & Bartley, 1947; Smith & Williams, 1948).

Quantitative measurements of free and total sulphadimidine have been made on excreta, and conjugated sulphadimidine calculated from the differences between these. It has not been possible to identify N^4-acetylsulphadimidine in the excreta by classical methods on account of the very small quantities available; however, identity in chromatographic behaviour in three solvent mixtures between one of the excretion products and the *N*-acetyl compound is strong evidence of identity.

EXPERIMENTAL

Insects. Locusta migratoria migratorioides (1–2 g.) were obtained from the Anti-Locust Research Centre, London, and were kept till required in glass-fronted wooden boxes with false zinc-gauze floors of the type recommended by the Anti-Locust Research Centre (see also Chauvin, 1941). They were maintained at 30–53° and relative humidity 60–70%.

The stock locusts were fed *ad lib.* on freshly cut grass, and on this diet immature adults produced daily about 0·5 g. of faecal pellets consisting mainly of undigested food residues. These were encrusted with the solid malpighian excretions (see Uvarov, 1948), which could only be separated partially with difficulty from the bulky vegetable material.

Administration of compounds. The locusts would not feed readily on green vegetables, but after a few days deprivation they would eat small amounts of moistened biscuit ('cream crackers') or rice paper, and in some qualitative feeding experiments it was convenient to spread the compound to be fed on rice paper or to incorporate it (1–5%) into a wet mash of broken biscuit. On these diets the droppings of hoppers and immature adults were very few and appeared to consist almost entirely of the gritty pink, malpighian excretions and could be worked up free from the large amounts of pigment and carbohydrate associated with the grass diet.

In quantitative experiments, compounds were administered in solution by injection into the thorax from an Agla micrometer syringe fitted with a no. 16 needle. Alternatively, a curved blunt needle could be used and the solution injected directly into the crop. The locusts, in general, tolerated injections of acid solutions, pH 3–7, well, but alkaline solutions, pH 8, were fatal. For feeding into the crop, the syringe was clamped at 45° to the horizontal with the needle pointing upwards and the locust held in the hand; with a little practice the needle could be inserted easily through the mouth into the crop without damaging the insect. When handled, locusts usually expelled a drop of crop fluid. This was removed with filter paper from the mouth parts and then 0·01 ml. of liquid could be introduced from the syringe and was retained by the locust.

In some quantitative experiments the sulphadimidine was dissolved in acetone and a measured amount of the solution placed on to a blade of grass on which the locust was allowed to feed. When enough had been consumed the insect was removed and the sulphadimidine remaining on uneaten material estimated colorimetrically. The amount eaten could then be obtained by difference. In most experiments, however, it was found more convenient to feed the locusts with the blunt needle.

The micrometer scale of the Agla syringe was not used as a direct measure, but volumes equal to those injected were taken during each experiment and estimated colorimetrically to find the exact amount administered.

After administering the drug, the locusts were confined, either singly or in groups, in 500 ml. beakers closed with paper covers and kept in a cage at 35–40° and 60–70% r.h. The droppings were collected periodically and the locusts transferred to fresh beakers. They were fed daily on a limited amount of grass (0·1–0·2 g./locust). Some grass was necessary to form the faeces as the locusts when starved did not completely empty their intestines. On the other hand, the amount of grass fed had to be as small as possible in order to reduce the interfering brown colours of the blanks in the diazo reaction.

Quantitative estimation of conjugated sulphadimidine. The difference between the 'free' and 'total' sulphadimidine excreted represented conjugated sulphadimidine including acetylsulphadimidine. 'Free' sulphadimidine was estimated by the method of Bratton & Marshall (1939). The faecal pellets were stirred in an Atomix blendor with a suitable known volume of 0·1 N-NaOH, filtered under pressure from any grass residues and samples of the filtrate taken for analysis. Blanks were made up from corresponding volumes of the filtrate treated in the same way, except that no naphthylethylenediamine was added. 'Total' sulphadimidine was estimated after acid hydrolysis as previously described (Smith & Williams, 1948) using a similarly treated sample of the faecal extract, without naphthylethylenediamine, as a blank. Recoveries of sulphadimidine and acetylsulphadimidine added to normal locust excreta were of the order of 90–100%.

Results with adults and hoppers are summarized in Tables 1 and 2.

Chromatographic detection of acetylsulphadimidine. Six locusts were dosed orally by syringe with 1·5 mg. each of sulphadimidine in 0·1 ml. of 2 N-HCl and allowed to feed on wet biscuit. The excreta, collected at 24 hr. and 48 hr., were ground in a mortar and extracted twice with 2·0 ml. of 4:1 (v/v) acetone/water mixture. After filtration and

Table 1. *Free and total sulphadimidine in locust excreta*

Stage	Amount eaten (μg.)	1st day Free (μg.)	1st day Total (μg.)	1st day Conjugated (%)	2nd day Free (μg.)	2nd day Total (μg.)	2nd day Conjugated (%)	3rd–5th days* Free (μg.)	3rd–5th days* Total (μg.)	3rd–5th days* Conjugated (%)	Total in exuvia (μg.)	Recovery (%)	Conjugates (as % of recovered)
					After oral administration on grass								
5th instar	1690	532	592	10	260	324	20	32	84	62	60	62	16
	1670	220	224	2	68	88	23	40	84	53	63	28	17
	2510	1144	1144	0	220	252	13	20	48	58	61	60	4
	2470	576	600	4	208	236	12	52	64	19	59	39	7
Immature adult	400	60	64	6	84	100	16	Not measured, <80 μg./locust			—	41	12
	700	112	148	24	96	160	40				—	44	32
	1140	260	312	17	140	176	21				—	43	18
	1140	92	92	0	124	146	15				—	21	9
	1560	328	328	0	116	140	17				—	30	5
	1840	888	912	3	276	324	15				—	67	6
	2930	672	752	11	236	284	17				—	35	12
	Amount injected (μg.)				After injection (grass diet)								
5th instar hoppers	560	252	304	17	48	64	25	No excreta†			—	66	19
	560	284	396	28	52	84	38				—	86	30
	560	228	300	24	36	72	50				—	67	29
	560	48	68	29	No excreta†			188	228	18	—	53	20
	560	28	28	0				92	180	49	—	37	42
	670	No excreta†						116	196	41	60	38	41
	670							104	116	10	30	22	10
	670							100	124	19	138	39	19
	670	No excreta†			96	128	25	52	68	24	30	34	25

* Hoppers moulted on 3rd and 4th days.
† Locust moulted.

Table 2. *Free and total sulphadimidine in excreta of immature adult locusts after injection of 850 μg. of sulphadimidine (grass diet)*

(All quantities in μg.)

1st day Free	1st day Total	1st day Conjugated (%)	2nd day Free	2nd day Total	2nd day Conjugated (%)	3rd–7th days Free	3rd–7th days Total	3rd–7th days Conjugated (%)	8th–15th days Free	8th–15th days Total	8th–15th days Conjugated (%)	Recovery (%)	Conjugates (as % of recovered)
440	440	0	112	124	10	56	68	18	40	64	37	82	7
362	376	4	52	92	43	20	32	38	52	68	24	67	14
72	84	14	104	124	16	72	340	79	40	64	38	72	53
337	401	16	100	140	29	52	232	78	32	48	33	97	36
304	352	14	72	92	22	72	100	28	20	36	44	68	19

evaporation to dryness on the water bath, the extract was taken up in acetone and separated chromatographically on paper (Whatman no. 1).

The spots were developed by spraying with 1% salicylaldehyde in 25% (w/v) trichloroacetic acid which gave an immediate yellow spot with sulphadimidine. After heating at 120° for 30 min., both sulphadimidine and acetylsulphadimidine gave a brown spot with an intense yellow-green fluorescence in ultraviolet light.

Free and acetylated sulphadimidine were identified in the extract by comparison with the behaviour of the authentic materials run on the same paper (Table 3). The acetyl compound was present in the excreta of both immature adults and 5th instar hoppers on the first and second days after ingestion of sulphadimidine.

Acetylation of 6-amino-4-nitro-o-cresol in the locust. The compound, 0·66 mg. dissolved in 0·01 ml. 0·5 N-HCl, was administered orally to two immature adults. This dose had no apparent ill effect, nor did the same quantity injected into the thorax of two other locusts. The excreta, collected 24 hr. later, were extracted with hot 80% (v/v) aqueous ethanol, filtered from grass residues, and the concentrated filtrate examined chromatographically on Whatman no. 1 paper for 6-acetamido-4-nitro-*o*-cresol and for the compound fed (cf. Smith, Smithies & Williams, 1953). The extract contained a substance having the same R_F values as 6-acetamido-4-nitro-*o*-cresol in benzene-acetic acid-water, 1:1:2 (R_F=0·84); benzene-*n*-butanol-ammonia sp.gr. 0·880, 2:5:2(R_F=0·44), and *n*-butanol-acetic acid-water, 4:1:5 (R_F=0·95), and giving the same yellow colour when

sprayed with N-NaOH. Considerable amounts of unchanged amino compound were also detected on the chromatograms (R_F values, 0·56, 0·20 and 0·95, respectively).

Table 3. R_F *values of sulphadimidine and acetylsulphadimidine*

(Paper: Whatman no. 1; time of run, 4 hr.)

Solvent mixture (parts by volume)	R_F values: Sulphadimidine	R_F values: Acetylsulphadimidine
*iso*Propyl ether-formic acid-water, 5:1:1	0·35	0·59
Ethyl methyl ketone-water, 1:1	0·97	0·86
Ethyl methyl ketone-2N-ammonia, 1:1	0·47	0·20

DISCUSSION

The results obtained show that the locust, in common with a wide selection of animal species (see Schnitker, 1948), can acetylate the aromatic amino group.

Absorption of sulphadimidine from the intestine appears to be slow and the high 'free' values found on oral administration are undoubtedly due to unabsorbed sulphadimidine. The degree of conjugation of that material which is absorbed is very variable, values ranging from 0 to 80% being obtained, without significant difference between hoppers and adults.

In some early experiments it was observed that the total recoveries of administered material were very low, particularly when hoppers were fed just before the moult. Examination of the moulted skin showed that it contained only a small amount of material diazotizable after hydrolysis and that the detoxication did not involve deposition of waste material in the exuviae as has sometimes been suggested (cf. Wigglesworth, 1950). It seems that the low recovery in these cases is to be associated with the cessation of feeding near the moult; this allows the drug 2–3 days for absorption since no excreta are produced when the insects are not eating. The excretion of material once inside the locust is very slow and in prolonged experiments (cf. Table 2) the recovery is higher. Measurements were discontinued after 15 days, but even 24 days after feeding, weak diazo reactions were obtained on the excreta which indicated that about a third of the eliminated drug was conjugated.

After the acetylation of sulphadimidine had been demonstrated it seemed of interest to feed 6-amino-4-nitro-*o*-cresol since this is a major metabolite of the insecticide 4:6-dinitro-*o*-cresol (DNOC) in the rabbit. The former compound has a low toxicity to rabbits and the locusts tolerated doses of 0·66 mg. without ill effects, while DNOC has a LD_{95} in *Locusta* of about 0·02 mg./locust (Hopf, 1951). In the locust some of the material fed was further metabolized to its acetyl derivative as is the case in the rabbit (Smith *et al.* 1953).

SUMMARY

1. The ability of *Locusta migratoria* to acetylate an aromatic amino group has been examined.

2. A variable amount of sulphadimidine (2-(*p*-aminobenzenesulphonamido)-4:6-dimethylpyrimidine) is conjugated by the locust; N^4-acetylsulphadimidine has been identified in the excreta by paper chromatography.

3. Sulphadimidine is poorly absorbed and slowly excreted by the locust.

4. Acetylation of 6-amino-4-nitro-*o*-cresol, a possible metabolite of 4:6-dinitro-*o*-cresol, also occurs in the locust.

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Phosphorus Compounds in the Cell

4. THE INCORPORATION OF RADIOACTIVE PHOSPHORUS INTO LIVER CELL FRACTIONS

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In part 3 of this series (Davidson & Smellie, 1952*b*) a method was described for the accurate determination of the specific activities of the individual ribonucleotides of the ribonucleic acid (RNA) of the tissues of animals which had received radioactive phosphorus. This method has now been applied to the RNA of cell nuclei and of the various morphological fractions of the cell cytoplasm. Preliminary notes on this work have already been published (Davidson, McIndoe, & Smellie, 1951; Smellie & McIndoe, 1952) and a full account is given here.

METHODS

Biological. Albino rats from the departmental colony and chinchilla rabbits bred in the Department, were used in these experiments. Both received 50 μc. ^{32}P/100 g. body weight as carrier-free inorganic phosphate by intramuscular injection.

When rabbits were used, samples of blood (5 ml.) were withdrawn from the ear vein 2 hr. after injection of isotope in order that the specific activity of the blood inorganic phosphate might be determined.

Rats were killed by exsanguination under ether anaesthesia and the rabbits by cervical dislocation. The livers were perfused with cold 0·9% (w/v) NaCl solution, dissected out and chilled. The liver tissue was finely minced with scissors and pulped in 0·25 M-sucrose either in a Potter-Elvehjem (1936) homogenizer or, where larger amounts of material were involved, in an M.S.E. Nelco blendor (Measuring and Scientific Equipment Ltd., London, S.W. 1) with the blades replaced by a paddle. The duration of this process was controlled by repeated microscopical examinations to ensure maximal rupture of cells with minimal breakdown of nuclei. Rabbit liver proved more resistant to disintegration than did rat liver. The suspension was first strained through gauze and centrifuged at 600*g* to remove nuclei and any unbroken cells. The deposit was washed once with an equal volume of 0·25 M-sucrose and the first supernatant and washings combined.

Nuclei were isolated either from the sediment obtained in this way after blending at low speed in citric acid or from a separate sample of the original liver tissue. The citric acid method as described by McIndoe & Davidson (1952) was employed in either case.

The cytoplasmic supernatant material from the sucrose suspension was routinely examined microscopically to verify the absence of nuclei. Subsequent fractionation followed the pattern of Schneider (1948), the fields employed for the sedimentation of the various fractions being as follows: mitochondria, 8500*g* for 10 min.; microsomes, 25 000*g* for 60 min. The supernatant fluid after removal of the microsomes constituted the cell sap. Centrifugation was carried out in an International or M.S.E. refrigerated centrifuge fitted with a high-speed attachment.

In agreement with the observation of Muntwyler, Seifter & Harkness (1950) and of Schneider & Hogeboom (1951) it was found that the top layer of the mitochondrial sediment obtained as described above was loosely packed by comparison with the main bulk. It was decanted along with the supernatant fluid for further fractionation. The quality of the mitochondrial preparation was checked from time to time by adding Janus Green B to a concentration of 1/100 000 in the original suspension. The stained mitochondrial sediment when incubated at 38° for 5 min. turned red. This property enabled it to be distinguished sharply from microsomal material (Potter, Recknagel & Hurlbert, 1951).

Electron microscopy. Samples of the preparations of mitochondria and microsomes were fixed with OsO_4 in 0·25 M-sucrose for 15 min. and spun down. This material was washed twice with distilled water to remove all traces of sucrose. The resultant fixed suspensions were prepared for examination in the electron microscope by allowing a small drop of a suitable dilution of the specimen to evaporate to dryness on a prepared specimen mount, a standard polyvinyl formal ('Formvar') film on a Philips slotted film support. The uninterrupted area of 0·2 mm.^{2} available for examination in the electron microscope is much greater than that obtainable with most other types of mount, and in this case it was possible to ensure that a completely representative field was chosen for photography. The specimens were shadowcast with palladium (Williams & Wyckoff, 1945) at an angle of 15°. The shadow length in the micrographs is thus approximately 3·5 times the particle height.

Chemical. The main bulk of the mitochondrial, microsomal and cell sap fractions was suspended in distilled water and treated with 0·5 vol. 30% (w/v) trichoroacetic acid (TCA). A sample of whole cytoplasm was likewise treated with TCA and from this acid extract inorganic phosphate

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Table 1. *Specific activities in counts/min./100 μg. P of the nucleotides of the RNA from the cytoplasmic fractions and nuclei of the liver tissue of a group of rats which had received* ^{32}P *2 hr. before being killed*

	Whole cytoplasm	Mitochondria	Microsomes	Cell sap	Nuclei
Adenylic acid	383	289	189	650	5920
Guanylic acid	201	182	109	303	4800
Cytidylic acid	360	215	141	575	5000
Uridylic acid	286	232	119	435	4870
DNA fraction	—	—	—	—	140

was precipitated as described by Davidson, Frazer & Hutchison (1951) and used for the determination of specific activity.

The material precipitated by TCA from all four fractions was washed twice with 10% (w/v) TCA and treated with lipid solvents. From the protein residue RNA was extracted by 'procedure 2' of Davidson & Smellie (1952*b*), hydrolysed with alkali and separated into the component ribonucleotides by ionophoresis on paper (Davidson & Smellie, 1952*a*). Portions of the nucleic acid hydrolysates were in some cases also used for determination of the base ratios as described in the following paper (Crosbie, Smellie & Davidson, 1953).

Nuclear RNA was obtained as a solution of mixed ribonucleotides by the modification of the Schmidt & Thannhauser (1945) procedure described by McIndoe & Davidson (1952). These were also separated by ionophoresis on paper.

The general methods employed in the assay of radioactive materials were those of Davidson, Frazer & Hutchison (1951).

The results of radioactivity measurements are expressed in terms of counts/min./100 μg. P. In the experiments on rabbits it was felt desirable to have a common denominator to which all specific activities might be related, especially when the results obtained at different time intervals were being compared. The specific activity of the blood inorganic phosphate at 2 hr. was accordingly determined and used for the calculation of the relative specific activity (R.S.A.). Where groups of rats were employed this method was not practicable. In such cases, when it was necessary to compare one series of experiments with another at the same time interval, results were expressed relatively to the tissue inorganic phosphate at the time of killing.

In the experiments on regenerating rat liver partial hepatectomy was carried out by the method of Higgins & Anderson (1931).

RESULTS

Electron micrographs of mitochondria and microsomes illustrated in Pl. 1 indicate that each of these fractions is relatively homogeneous in the morphological sense, and that the two fractions differ markedly from each other in the size of the component particles. The diameters of the mitochondria range from 410 to 860 mμ. with a mean value of 550 ± 90 mμ. and those of the microsomes from 10 to 200 mμ. There is no appreciable overlap in the sizes of the two groups. The mitochondria are, on the whole, circular or nearly circular in outline, and the individual particles throw large shadows indicating that the mitochondria themselves are nearly spherical. The particle density after osmic acid fixation is such that there is little transmission of an 80 kV. electron beam and there is no suggestion of internal structure. In contrast, the photographs of microsomes show a rather wide array of particle sizes and the individual particles are in every case irregular in outline. The large particles invariably throw short shadows and the impression gained from the micrograph is that the material consists of varying sizes of aggregates of a component of about 10 mμ. particle diameter.

The specific activities of the ribonucleotides from the RNA of various parts of the rat-liver cell are shown in Table 1 for an experiment of 2 hr. duration. It is clear that the RNA of the nucleus is very much more active than that of any of the cytoplasmic fractions. Of these fractions themselves the cell sap gives values about twice as high as those obtained from the granules. We have invariably found in many experiments of this duration in rats and rabbits that the ribonucleotides from the microsomes are slightly less active than those from the mitochondria. The figures for the whole cytoplasm, as might be expected, are intermediate between those for microsomes and cell sap.

There appears to be no great difference in specific activity between the four ribonucleotides from any one source, but adenylic acid is generally slightly higher and guanylic acid slightly lower in activity than the pyrimidine nucleotides.

In Table 2 the results of an experiment of 4 hr. duration are given in terms of R.S.A. with tissue inorganic phosphate taken as 1000. The pattern for the control animals which were submitted to the sham operation is the same as for the normal animals shown in Table 1, although the values for mitochondria and microsomes lie very close together. The regenerating livers, however, present some differences from the controls. The nuclear ribonucleotides again show very high activities which are about twice those found in the control animals. The activities of the ribonucleotides from the whole cytoplasm are also raised to values about 3 or 4 times those in the controls. The rise in the activity of the ribonucleotides in the whole cytoplasm is reflected in each of the three fractions. Of the cytoplasmic fractions the cell sap still gives the highest figures while mitochondria and microsomes are almost equal. The nucleotides in each fraction again show only slight differences among themselves, adenylic acid as usual showing the highest values.

Table 2. *Specific activities (counts/min./100 μg. P) relative to tissue inorganic phosphate as 1000, of ribonucleotides from cytoplasmic and nuclear RNA and of DNA from the livers of partially hepatectomized rats* (R) *(regenerating 26 hr.), of sham operated rats* (C) *and of weanling rats* (W)

(32P was administered 4 hr. before killing.)

	Whole cytoplasm			Mitochondria			Microsomes			Cell sap			Nuclei		
	R	C	W	R	C	W	R	C	W	R	C	W	R	C	W
Phospholipin	402	207	480	366	213	416	422	242	511	396	278	478	370	211	385
Adenylic acid	83	24	39	75	19	29	74	16	33	98	35	49	590	361	370
Guanylic acid	69	16	27	51	13	22	64	12	21	80	23	33	606	318	349
Cytidylic acid	74	23	37	69	16	25	59	14	20	94	41	50	590	320	338
Uridylic acid	72	21	33	67	19	21	62	12	20	90	31	38	702	308	380
DNA fraction	—	—	—	—	—	—	—	—	—	—	—	—	43	3	14

Table 3. *Specific activities (counts/min./100 μg. P), relative to the adult blood inorganic phosphate at 2 hr. as 100 000, of phospholipins, of ribonucleotides from cytoplasmic and nuclear RNA and of DNA from the livers of rabbits 4 hr. after receiving* 32P

(N, normal female; M, pregnant female; F, foetus.)

	Whole cytoplasm			Mitochondria			Microsomes			Cell sap			Nuclei		
	N	M	F	N	M	F	N	M	F	N	M	F	N	M	F
Phospholipin	1860	2730	1750	1430	—	—	1710	—	—	1710	—	—	1480	3250	1910
Adenylic acid	485	559	1090	341	361	389	212	126	628	495	861	1520	5990	9160	8970
Guanylic acid	450	256	940	308	276	277	162	134	540	302	432	1160	4890	8660	7440
Cytidylic acid	323	423	855	252	290	324	151	213	558	438	610	1230	5670	7960	7580
Uridylic acid	456	572	1020	307	340	328	148	147	696	438	830	1422	4530	8820	10250
DNA fraction	—	—	—	—	—	—	—	—	—	—	—	—	273	600	3260
Tissue inorganic phosphate	33600	73100	42900	—	—	—	—	—	—	—	—	—	—	—	—

Figures for the specific activities of the phospholipins are also given in Table 2. There is no great difference between nuclear and cytoplasmic material, but the activity in all fractions is greater in the regenerating liver than in the controls.

As might be expected, the deoxyribonucleic acid (DNA) in the nuclei of the control livers shows a very low activity. In the regenerating livers, on the other hand, in which cell division is active the specific activity is increased about 15-fold.

In the weanling rats, which were all about 50 g. body weight, the specific activity of the DNA, as might be expected for growing tissue, is about 5 times greater than in the controls, giving a value intermediate between those for resting and regenerating adult livers. Values for phospholipin are of the same order as those for regenerating liver. The activities of the ribonucleotides follow the same general pattern as in the sham-operated rats but are in all cases slightly higher.

The results of an experiment on maternal and foetal liver obtained from a pregnant rabbit are compared in Table 3 with those from a normal female animal. These results are expressed as R.S.A. in terms of the adult-blood inorganic phosphate at 2 hr. as 100000, but they may also be expressed relatively to the tissue inorganic phosphate of the liver concerned by using the data given in the last line of the table.

The pattern found for the normal rabbit is similar to that obtained for the rat. In the pregnant animal the specific activities of the nuclear ribonucleotides and phospholipins are increased as compared with the normal animal. This also holds for the ribonucleotides of the cell sap, but not for the granules. The figure for the specific activity of DNA is also slightly raised.

Conditions in the foetus, however, are very different. The specific activity of the DNA is high; phospholipins both in nuclei and cytoplasm show slightly lower values than are given by maternal liver, while nuclear ribonucleotides give figures of the same order as are found in the maternal tissue.

The specific activities for the ribonucleotides of whole cytoplasm are much higher than those found in normal or maternal liver, and these differences are due to the high values found in the cell sap and microsomes, especially the latter. Figures for mitochondria are of the same order as those for maternal liver.

Whereas in normal and maternal rabbit liver (and also in normal, regenerating and weanling rat liver) the specific activity of the ribonucleotides from the mitochondria was invariably of the same order as, or slightly higher than, that of the ribonucleotides from the microsomes, in foetal liver the situation is reversed and the microsomes give values considerably higher than those for the mitochondria,

although appreciably lower than those found in the cell sap.

If the results are expressed relatively to the tissue inorganic phosphate there is no change in the relationships between the fractions from any one type of liver, but the relationships of the various types of liver to each other are slightly altered. For example, the activities of the nuclear ribonucleotides from normal and maternal liver become closely similar while those for the foetal liver are much higher. In the cytoplasm the normal figures become higher than the maternal while those for the foetus remain higher than both.

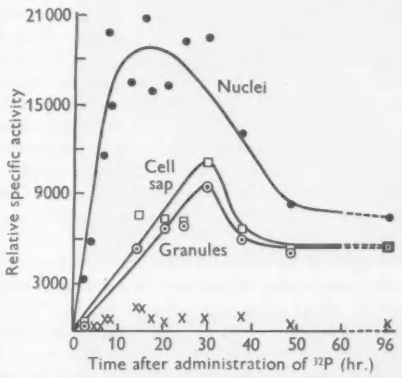


Fig. 1. Specific activity (counts/min./100 μg. P) relative to the specific activity of blood inorganic phosphate, 2 hr. after administration of the isotope, of the cytidylic acid from the RNA of cytoplasmic granules (⊙), cell sap (□) and nuclei (●) of rabbit-liver tissue at varying intervals after the administration of ^{32}P. The mitochondria and microsomes gave values so close together that they have been combined as 'granules'. The crosses represent the specific activities of DNA.

The results which have so far been described were obtained from animals which were killed 2 or 4 hr. after administration of ^{32}P, but experiments have also been carried out with rabbits in which time intervals ranging from 2 to 96 hr. were employed with the results shown in Fig. 1.

These curves were obtained from the cytidylic acid of the nuclear and cytoplasmic RNA's. The other three nucleotides give similar patterns. At time intervals greater than 4 hr. the figures for mitochondria and microsomes lie so close to each other that these two fractions have had to be represented together as 'granules'. Figures for cell sap are consistently slightly higher than those for granules and reach a maximum value at about the same time (approx. 30 hr.) after administration. Values for nuclear cytidylic acid rise very steeply to a maximum of about twice that found for cytoplasmic nucleotides. Although the figures show a fairly wide scatter, it is clear that the maximum occurs earlier (about 15 hr.) for nuclear cytidylic acid than for cytoplasmic material.

Even at 96 hr. the figures for nuclear and cytoplasmic material remain surprisingly high, but the value for nuclear cytidylic acid is still higher than that for cell sap or granules.

The specific activity of the DNA is at all time intervals low and shows no evidence of a progressive rise with time.

DISCUSSION

In any process of fractionation of cellular components one of the first considerations must be the technique used for the breakdown of the cells. The Waring type of blender produces such powerful shearing forces as to cause disruption of cellular components (in particular nuclei) in a sucrose medium. The Potter-Elvehjem (1936) glass homogenizer is more suitable for the purpose but does not readily handle large amounts of material. In addition, a small quantity of powdered glass is produced which can prove troublesome in the subsequent analysis. We have therefore used a Potter-Elvehjem type of homogenizer with a Perspex pestle (Potter, 1949). For large amounts of material the rotating paddle has proved to give good disruption of cells in sucrose without undue destruction of nuclei.

Microscopic examination of these suspensions has shown that a very large number of free and apparently undamaged nuclei are still present, together with a few whole cells. The presence of these whole cells is inevitable unless disintegration is considerably prolonged with a resultant breakdown of large numbers of nuclei. Confirmation of the absence of large numbers of broken nuclei lies in the fact that the DNA content of the whole cytoplasm, after removal of the nuclear fraction, has been found to be negligible. Since the ratio RNA:DNA in the nucleus is not more than 1:5 it is reasonable to suppose that there is little contamination of cytoplasmic RNA with RNA derived from broken nuclei, although it is not impossible that some RNA may have been leached out of the intact nuclei during the initial stages of fractionation.

It is generally accepted that differential centrifugation of suspensions of liver pulp in sucrose yields particles of two main types, large particles corresponding to the mitochondria and small particles corresponding to the microsomes (Schneider & Hogeboom, 1951), although it has been claimed by Chantrenne (1947) that the range of heterogeneity is much greater than can be accounted for by this simple classification. Chantrenne (1947), however, used a buffered saline solution in which the mitochondria and microsomes tend to agglutinate

(Schneider & Hogeboom, 1951), and this might possibly explain the spectrum of particles he observed.

Using suspensions of liver pulp in 0·88M-sucrose, Novikoff, Podber, Ryan & Noe (1952) separated eight fractions, three of which were essentially mitochondrial, two were mixtures of mitochondria and microsomes and three were essentially microsomal. They concluded that the fundamental products of differential centrifugation are mitochondria and large and small microsomes. The latter would presumably correspond to the microsomes of different sizes shown in Pl. 1. In our experience these microsomes, of whatever size, failed to give the characteristic reaction with Janus Green B which was observed in the mitochondria.

The evidence obtained from electron microscope examination (Pl. 1) is strongly in support of the view that there are two varieties of cytoplasmic particles, the mitochondria which appear as very well-defined spherical granules and the microsomes which are less homogeneous in appearance. On the basis of structure alone it is possible to identify the components from each fraction in artificially mixed samples since the large structures contributed by the microsomal preparations, as shown in the lower picture in Pl. 1, are clearly loose aggregates of smaller particles, whereas the granules contributed by the mitochondrial preparations are distinct spherical entities. As with all morphological work with fixed material in the electron microscope, the possibility of fixation artifacts cannot be completely eliminated, but care was taken in these experiments to ensure that both fractions were given the same treatment during fixation. It is therefore reasonable to assume that the structural differences observed represent real distinctions between the two fractions. For this reason, however, the study of the detailed morphology of the large granules themselves is made difficult in the present state of knowledge of fixative action on nucleoprotein-containing structures. The observations made on partially osmolysed granules would support those reported by Claude & Fullam (1945) and by Mühlethaler, Müller & Zollinger (1950).

The determination of the specific activities of protein-bound phosphorus compounds is well known to present serious difficulties owing to the presence of highly labelled non-nucleotide materials (Davidson, Frazer & Hutchison, 1951). The method of ionophoresis, which has been employed in these experiments, is known to overcome these difficulties and to provide true values for the specific activities of the ribonucleotides (Davidson & Smellie, 1952*b*). From the results in Tables 1–3, it is clear that the incorporation of ^{32}P into RNA is of the same order in all four nucleotides from any one subcellular component, although the activity of adenylic acid tends to be slightly higher and of guanylic acid slightly lower than those of the pyrimidine nucleotides. This is in agreement with the observations of Volkin & Carter (1951). The high figures for uridylic acid quoted by Davidson, McIndoe, & Smellie (1951) are due to the presence of other components of high activity which have been described by Davidson & Smellie (1952*b*). The presence of these constituents is almost certainly the cause of the high figures for uridylic acid mentioned by Hultin, Slautterback & Wessel (1951).

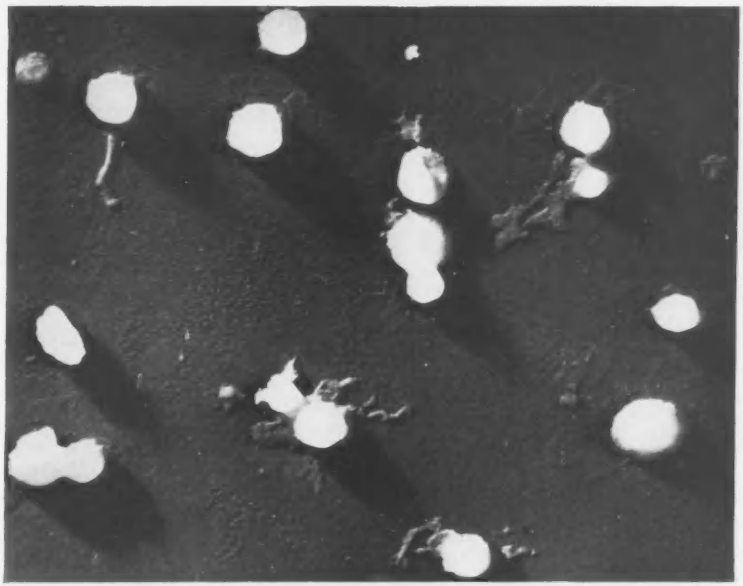
The very high specific activity of the ribonucleotides from nuclear RNA is consistent with the results of other authors whose work has recently been reviewed by McIndoe & Davidson (1952).

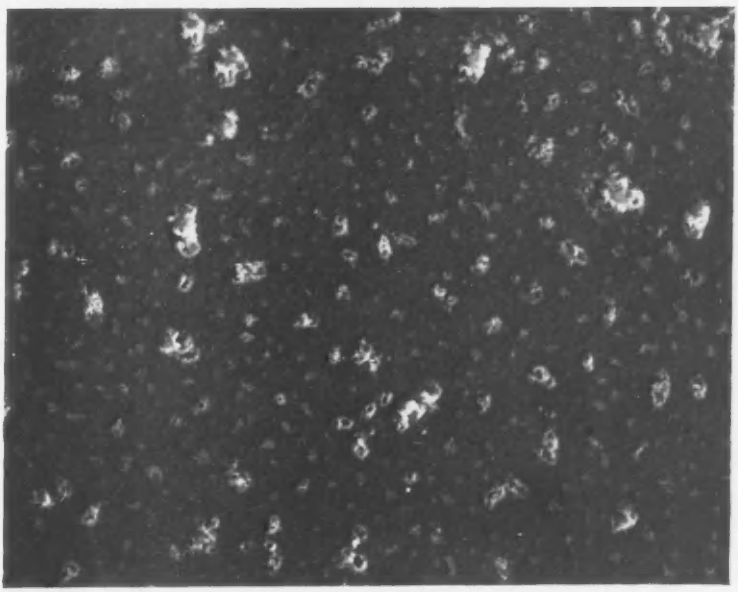
We have invariably found with time intervals of 2 or 4 hr. in normal rat or rabbit liver that the specific activity of the ribonucleotides from the microsomes is of the same order as, or slightly lower than, that of the ribonucleotides from the mitochondria both in sucrose and in saline suspensions. Figures for the cell sap are always higher than those for the granules. The possibility cannot be excluded that this high figure is due in part at least to the presence in this fraction of some RNA which has been leached out of the nuclei.

The differences between the specific activities for the ribonucleotides of the mitochondria and microsomes at short time intervals is small. With increasing time the specific activities of the nucleotides from mitochondria and microsomes are greatly increased without a proportionate increase in the difference between them, so that this difference tends to become negligible.

Marshak & Calvet (1949), using rabbit liver, found a higher value for the specific activity of small cytoplasmic granules than for large granules at varying time intervals between 1 and 73 hr. after administration of ^{32}P, but their granules were obtained from a suspension of liver pulp in 5 % (w/v) citric acid. Specific activity measurements were made on the bulk nucleotide fraction obtained by the method of Schmidt & Thannhauser (1945). This fraction is known to contain contaminating phosphorus compounds of high specific activity (Davidson, Frazer & Hutchison, 1951; Davidson & Smellie, 1952*b*).

Jeener & Szafarz (1950), using the livers of rats 2 hr. after administration of ^{32}P, found a higher specific activity in their small granule fraction (pellet, $6 \times 10^4 g$ for 60 min.) than in their large granule fraction (pellet, $1{\cdot}3 \times 10^4 g$ for 10 min.). In mouse embryo this situation was reversed. The RNA of the supernatant fluid invariably showed higher activity than any of the granules. It should be emphasized that Jeener & Szafarz (1950) disintegrated their tissue in a Waring type of homogenizer and centrifuged it in phosphate buffer under centrifugal fields considerably higher than those employed





Electron micrographs of cytoplasmic particles prepared by differential centrifugation from a suspension of rat-liver tissue in 0·25 M-sucrose. Magnification × 15 000. Shadowed at 15° with palladium. Upper picture, mitochondria; lower picture, microsomes.

R. M. S. SMELLIE, W. M. McINDOE, R. LOGAN, J. N. DAVIDSON AND I. M. DAWSON—PHOSPHORUS COMPOUNDS IN THE CELL. 4. THE INCORPORATION OF RADIOACTIVE PHOSPHORUS INTO LIVER CELL FRACTIONS.

by us. It is therefore scarcely justifiable to compare their results with ours, but it seems possible that the high centrifugal fields which they employ yield a large-granule fraction which is a mixture of mitochondria and microsomes and a microsome fraction which contains some of the larger molecules of ribonucleoprotein from the cell sap. This tendency would be emphasized by their use of phosphate buffer in place of sucrose as the suspending medium.

Barnum & Huseby (1950), using saline suspensions of the livers of mice which had received ^{32}P, found no appreciable difference between the R.S.A. of isolated RNA from mitochondria and microsomes. They did, however, observe that the specific activity of the phospholipin in mitochondria was significantly lower than that of the microsomes. A similar observation has been recorded by Ada (1949) and is in agreement with our own results (Table 2).

In two types of liver tissue in which cell division is occurring, regenerating rat liver and the livers of weanling rats, the same general pattern appears as is found in normal rat liver although the specific activities are in general higher than for normal tissue (Table 2). High values for the specific activities of RNA in regenerating rat liver have also been recorded by Khesin (1951) and Johnson & Albert (1952).

In the maternal rabbit liver, as compared with the normal, the high specific activity of the ribonucleotides is particularly noticeable in the cell sap while the values for the granules are almost unchanged (Table 3). The foetal liver is unique in that the activity in the microsomes is increased disproportionately so that the value exceeds that of the mitochondria. This is the reverse of the results obtained by Jeener & Szafarz (1950) for mouse embryo.

The specific activity of the DNA is increased in weanling rat liver, a moderately rapidly growing tissue, and greatly increased in rat liver regenerating 26 hr. after partial hepatectomy (Table 2). At this stage cell division is very active. The ratios of the specific activities of RNA from whole cytoplasm to nuclear DNA are about 7:1 for normal rat liver and 1·7:1 for regenerating liver. These ratios agree well with figures previously quoted for whole isolated nucleic acids by Davidson (1947). The corresponding figure for normal rabbit liver is 1·6:1. The approximately twofold increase in specific activity of DNA from pregnant-rabbit liver over that for normal rabbit liver (Table 3) is in agreement with the results of Kelly, Payne, White & Jones (1951) for rat liver.

In the foetal rabbit liver the specific activity of the DNA is increased about tenfold in comparison with the adult tissue, while that of the RNA is approximately doubled. The result is that the activity of RNA is considerably lower than that of the DNA (ratio 0·3:1). Ratios less than unity have already been recorded by Davidson (1947). In the sea-urchin embryo Villee, Lowens, Gordon, Leonard & Rich (1949), using ^{32}P, and Abrams (1951), using [^{14}C]glycine, have shown that the specific activity of DNA is much higher than that of RNA, without distinguishing the intracellular location of the RNA.

The results shown in Fig. 1 emphasize the differences between nuclear and cytoplasmic RNA. The nuclear material not only incorporates ^{32}P more readily but the uptake reaches a maximum earlier than in cytoplasmic RNA. Our experiments on rabbits show a maximum incorporation into nuclear RNA about 15 hr. after administration. The corresponding maximum for the nuclear RNA of mouse liver incorporating ^{32}P was found by Barnum & Huseby (1950) to be about 2 hr. after administration, while Hurlbert & Potter (1952) found the maximum incorporation of radioactive orotic acid by nuclear RNA in the rat to occur about 4 hr. after administration.

Marshak & Calvet (1949) quote a few figures indicating maximum incorporation of ^{32}P into rabbit-liver nuclear RNA between 2 and 12 hr. after administration.

Mauritzen, Roy & Stedman (1952), discussing nuclear RNA in a recent paper, state that they are unable to decide whether the RNA of isolated cell nuclei is a true nuclear component or an impurity adsorbed from the cytoplasm. Our results make it clear that the nucleotides of rabbit-liver nuclear RNA incorporate ^{32}P about 6 times more rapidly than do the nucleotides of cytoplasmic RNA from any fraction. These results confirm the discrete nature of nuclear RNA.

Jeener & Szafarz (1950), on the basis of their experiments with radioactive phosphorus, have suggested that RNA after passing from the nucleus into the cytoplasm appears first in a non-sedimentable form and that it is then built up into the microsomes and so into the mitochondria. If this hypothesis were correct three separate specific activity/time curves would be expected with maxima in the order: cell sap, microsomes and mitochondria. Our experiments on ^{32}P incorporation into the RNA's of rabbit liver at different time intervals are not altogether compatible with this proposal since the activity/time curves of the RNA's from the three cytoplasmic fractions are closely similar.

Our results do not in any way preclude the possibility that nuclear RNA serves as the ultimate precursor of cytoplasmic RNA's, although the activity/time relationships which we observed (Fig. 1) are not consistent with a process of simple diffusion from the nucleus into the cytoplasm. Such a process moreover would be expected to yield cytoplasmic RNA of the same molar composition

as that of nuclear RNA whereas marked differences have been observed (McIndoe & Davidson, 1952; Crosbie *et al.* 1953). The specific activity/time relationships of the RNA's from the cytoplasmic particles suggest that they are being synthesized at the same rate from a common precursor. The differences between the cytoplasmic particles and the cell sap are sufficiently small to be explained on the basis of permeability of the particles or of slight contamination of the cell sap with nuclear RNA.

SUMMARY

1. Using the technique of differential centrifugation in 0·25 M-sucrose, rat- and rabbit-liver cell cytoplasm have been separated into two particulate fractions and a non-sedimentable supernatant fluid corresponding to the cell sap. Isolated cell nuclei have also been prepared.

2. The particulate fractions correspond to the mitochondria and microsomes and have been characterized by Janus Green staining and by electron microscopy which has shown them to consist of two independent populations of particles.

3. The incorporation of ^{32}P into the phospholipins and ribonucleic acid (RNA) of the isolated nuclei and three cytoplasmic fractions and into the deoxyribonucleic acid (DNA) of the isolated nuclei has been studied in normal, regenerating and weanling rat liver and in normal, maternal and foetal rabbit liver. In all cases the most rapid incorporation was found in nuclear RNA. In the cytoplasm the cell sap invariably showed a higher activity than the granules. In foetal rabbit liver the microsomes showed a higher activity than the mitochondria. In all the other forms of liver tissue studied the activity of the microsomes was always of the same order as or slightly lower than that of the mitochondria.

4. The incorporation of ^{32}P into RNA in the various morphological fractions of normal rabbit liver has been determined at various time intervals. Nuclear RNA showed maximum incorporation about 15 hr. after administration whereas the three cytoplasmic fractions all showed maximum incorporation at about 30 hr.

5. Incorporation of ^{32}P into DNA in resting liver tissue was very low. In rat liver, regenerating after partial hepatectomy, the activity of the DNA was greatly increased, and in foetal rabbit liver was raised to a value much higher than that of the cytoplasmic RNA.

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Phosphorus Compounds in the Cell

5. THE COMPOSITION OF THE CYTOPLASMIC AND NUCLEAR RIBONUCLEIC ACIDS OF THE LIVER CELL

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It is well known that ribonucleic acid (RNA) is found in the cell cytoplasm both in the large and in the small particles and in the non-sedimentable supernatant fluid (the cell sap). RNA also occurs in the cell nucleus in small amounts (for review of literature see McIndoe & Davidson, 1952). It is clearly a matter of some interest to know whether variations occur in the RNA from these different sites. In this paper an attempt is made to determine the composition of RNA from different parts of the liver cell using the method of ionophoresis of nucleotides described in part 2 of this series (Davidson & Smellie, 1952*a*). This method is contrasted with the chromatographic procedure in common use after acid hydrolysis of the nucleic acid.

METHODS

Biological. Albino rats from the Department's colony and chinchilla rabbits bred in the Department were used in these experiments. The fractionation of liver tissue into mitochondria, microsomes and cell sap was carried out as described by Smellie, McIndoe, Logan, Davidson & Dawson (1953).

Nuclei were isolated from samples of the same liver tissue by the citric acid method as described by McIndoe & Davidson (1952).

Chemical. RNA was extracted from the cytoplasmic components by 'procedure 2' of Davidson & Smellie (1952*b*). Nuclear RNA was obtained as a solution of mixed ribonucleotides by the modification of the Schmidt & Thannhauser (1945) method described by McIndoe & Davidson (1952). Yeast RNA, supplied by British Drug Houses Ltd., was purified by the method of Smith & Markham (1950) followed by precipitation from glacial acetic acid. Whole-liver RNA was isolated by the method of Davidson, Frazer & Hutchison (1951).

The following methods of analysis of RNA were employed:

Method (*a*). The nucleic acid (10 mg.) was digested to the constituent bases with 0·1 ml. 72% (w/w) $HClO_4$ at 100° for 1 hr. (or other suitable time) (Marshak & Vogel, 1951; Wyatt, 1951). The hydrolysate was diluted to 1 ml. and 20–30 μl. portions were applied to Whatman no. 1 paper from a micrometer syringe for separation by paper chromatography using 65% aqueous *iso*propanol 2N with respect to HCl. The following R_F values were obtained: guanine 0·33, adenine 0·48, cytosine 0·57, uracil 0·73, but these were subject to slight variation from run to run.

The spots were located by the procedure of Holiday & Johnson (1949), cut out and eluted by shaking vigorously in glass-stoppered test tubes with 5 or 10 ml. HCl of appropriate concentration as indicated in Table 1. The tubes were incubated at 37° overnight and the contents centrifuged. Readings of optical density were taken in the Beckman Model DU spectrophotometer. A separate lane of paper was cut up and eluted for use as blanks. Each digest was run, eluted and read in triplicate.

Method (*b*). The nucleic acid was hydrolysed to purine bases and pyrimidine nucleotides by heating 20 mg. quantities with 1 ml. N-HCl for 1 hr. at 100° (Smith & Markham, 1950). Separation of components of the hydrolysate was carried out as in (*a*) above, the following R_F values being obtained: guanine 0·33, adenine 0·48, cytidylic acid 0·76, uridylic acid 0·91. Elution and ultraviolet absorption measurements were carried out as in method (*a*).

Method (*c*). The nucleic acid (10 mg.) was hydrolysed to the constituent ribonucleotides with 1 ml. 0·3N-KOH. The

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Table 1. *Ultraviolet absorption data for purine and pyrimidine derivatives showing the millimolecular extinction coefficient* ($m\epsilon$) *and* $E^{0.001\%}_{1\,cm.}$

	Concentration of HCl (N)	Wavelength (mμ.)	$m\epsilon$	$E^{0.001\%}_{1\,cm.}$ (found)	$E^{0.001\%}_{1\,cm.}$ (literature)	
Adenine	0·1	262·5	13·0	0·96	0·96	(Wyatt, 1951)
Guanine	1·6	249	10·9	0·72	0·73*	(Wyatt, 1951)
Cytosine	0·1	275	11·0	0·99	0·95	(Wyatt, 1951)
Uracil	0·1	259	7·9	0·702	0·705	(Wyatt, 1951)
Adenylic acid	0·01	260	—	—	0·401	(Volkin & Carter, 1951)
Guanylic acid	0·01	260	—	—	0·325	(Volkin & Carter 1951)
Cytidylic acid	0·01	278	—	—	0·393	(Ploeser & Loring, 1949)
Uridylic acid	0·01	260	9·89	0·305	0·305	(Ploeser & Loring, 1949)
Uridylic acid	0·1	260·5	—	—	0·308	(Volkin & Carter, 1951)

* 0·1N-HCl.

digest was acidified in the cold with 60% (w/w) $HClO_4$ to pH 3·6. Potassium perchlorate was removed by centrifugation and portions of the supernatant fluid dispensed on Whatman 3 MM paper strips 72 cm. long for ionophoresis as previously described by Davidson & Smellie (1952*a*). After the run the papers were dried, cut up and eluted. Phosphorus was determined in each eluate (Davidson & Smellie, 1952*a*).

Method (*d*). This was a modification of method (*c*) using strips of Whatman no. 1 paper. Quantities of hydrolysate corresponding to about 200 μg. total nucleotides were applied to the paper. The following relative mobilities were found:

	Relative mobility	Relative net charge/molecule at pH 3·6
Cytidylic acid	0·18	0·16
Adenylic acid	0·36	0·35
Guanylic acid *a*	0·77	0·83
Guanylic acid *b*	0·80	
Uridylic acid	1·00	1·00

Elution and ultraviolet absorption measurements were carried out as in method (*a*). A separate paper strip was run simultaneously to provide the blanks. It was invariably found that guanylic acid appeared as two closely adjacent bands both having the typical ultraviolet absorption spectrum of guanylic acid. We therefore consider them to represent the *a* and *b* isomers of Cohn (1951), but not necessarily respectively. They are labelled in the order indicated by analogy with their behaviour on an anion-exchange column. The relative mobilities of all the nucleotides quoted were calculated after allowance had been made for electro-endosmotic flow. Comparison of these mobilities with the relative net charge/molecule calculated from the data of Cohn (1950) shows good agreement.

In the methods (*a*) and (*b*) readings of optical density were made at the absorption maximum and also at another appropriate wavelength in order to determine the difference (Δ) in optical density as recommended by Vischer & Chargaff (1948*a*). For purposes of calculation, standard Δ values (Δ_{st}) were obtained from solutions of appropriate materials at concentrations of 10 μg./ml. in the same solvents as were used for elution (Table 2). The ultraviolet absorption data shown in Table 1 were used as criteria of purity of the bases and in the estimation of the nucleotides separated in method (*d*). In this last method absorption measurements were made at the maximum only.

The molar extinction coefficients for the nucleotides used are for the equilibrium mixtures of the *a* and *b* isomers formed in acid solution. The same acid was used for elution. This ensures that whatever may be the relative proportions of the *a* and *b* isomers of each of the nucleotides on the paper, the eluates always contain the same equilibrium mixture as that used in determining the molar extinction coefficients. This eliminates any possibility of error which arises out of the reported differences in optical properties of the isomers (Brown, Dekker & Todd, 1952).

Identification of pentose residues. Portions of the hydrolysate obtained in method (*b*) above were concentrated *in vacuo* and suitable quantities applied to Whatman no. 1 paper, neutralized with ammonia vapour and subjected to descending chromatography using an aqueous ammoniacal ethyl methyl ketone solvent. The solvent was prepared by equilibrating ethyl methyl ketone (100 ml.) with 1% (w/v) NH_4OH (40 ml.) and using the upper layer as the organic phase (cf. Partridge & Westall, 1948). The spots were detected using aniline hydrogen oxalate (Partridge, 1949). The following R_G values were found: arabinose 2·0, xylose 2·5, lyxose 2·9, ribose 3·9.

RESULTS

As has previously been observed (Davidson & Smellie, 1952*a*) the results obtained by ionophoresis (method (*c*)) show higher values for uracil (and for cytosine) relatively to adenine than are obtained by separation of the bases after perchloric acid digestion (method (*a*)). This is illustrated in Table 3 for three specimens of RNA from different sources and for a mixture of nucleotides.

Table 2. *Standard values* (Δ_{st}) *for the difference in extinction at the absorption maximum and at another chosen wavelength for various compounds in concentrations of* 10 μg./ml. *in* HCl *solution*

	Concentration of HCl (N)	Absorption maximum (mμ.)	Other chosen wavelength (mμ.)	Δ_{st} found	Δ_{st} Vischer & Chargaff (1948*a*)
Adenine	0·1	262·5	290	0·935	0·900
Guanine	1·6	249	290	0·539	—
Cytosine	0·1	275	290	0·546	0·545
Uracil	0·1	259	280	0·609	0·590
Cytidylic acid	0·01	278	300	0·331	—
Uridylic acid	0·1	260·5	280	0·814	—

Table 3. *Analyses of ribonucleic acids by methods* (*a*) *and* (*c*) *expressed as molar ratios relative to adenine as* 10

	Mixture of nucleotides		Rat-liver RNA		Rabbit-liver RNA		Chick-liver RNA	
Method	(*a*)	(*c*)	(*a*)	(*c*)	(*a*)	(*c*)	(*a*)	(*c*)
Adenine	10	10	10	10	10	10	10	10
Guanine	9·3	9·1	17·5	17·6	16·5	16·9	17·1	17·1
Cytosine	10·7	11·0	13·6	14·3	14·5	14·6	12·4	13·6
Uracil	11·9	13·1	8·1	10·8	7·3	10·3	7·3	10·6

Table 4. *Analyses of two specimens of yeast ribonucleic acid by different methods expressed as molar ratios relative to adenine as* 10

(The results of triplicate analyses are given to illustrate the agreement between replicates.)

	Yeast RNA I			Yeast RNA II		
Method	(*a*)	(*b*)	(*d*)	(*a*)	(*b*)	(*d*)
Adenine	10	10	10	10	10	10
Guanine	11·4	11·6	11·3	11·1	11·3	11·0
	11·2	11·6	11·6	10·7	11·1	11·0
	11·5	11·7	11·6	11·3	11·0	11·0
Cytosine	8·0	9·0*	9·4	7·8	8·5*	9·4
	8·0	8·9*	9·7	7·9	8·6*	9·7
	8·1	9·0*	9·5	7·8	8·6*	9·6
Uracil	7·8	9·5*	10·2	8·8	10·7*	13·3
	7·9	9·1*	10·0	8·5	10·8*	13·2
	7·6	9·5*	9·9	8·8	10·3*	13·2
Calculated recovery of phosphorus (%)	86·2	88·5	97·1	—	—	—

* Corrected as described by Smith & Markham (1950).

Table 5. *Analyses of a specimen of yeast ribonucleic acid by method* (*a*) *with variations in the time of hydrolysis*

(Results expressed as molar ratios relative to adenine as 10.)

	Time of hydrolysis (min.)							
	15	30	45	60	120	180	240	360
Adenine	10	10	10	10	10	10	10	10
Guanine	11·2	11·3	11·1	11·4	11·1	11·2	11·3	11·5
Cytosine	5·7	7·1	7·2	8·0	8·8	8·6	8·2	8·9
Cytosine*	5·8	7·2	7·1	8·1	8·8	8·6	8·2	9·1
Uracil	6·2	7·4	7·4	7·8	8·4	7·8	7·9	7·7
Uracil*	10·4	9·9	9·5	8·9	9·2	8·5	8·3	8·3

* Calculated from optical density at absorption maximum and not from Δ values.

The analysis of one specimen of yeast RNA by three different methods is shown in Table 4. Comparison of the first and last columns again reveals the low value for uracil obtained after perchloric acid hydrolysis. When the nucleic acid was hydrolysed with N-hydrochloric acid at 100° for 1 hr., the values for uracil and cytosine were considerably raised even without the 5% correction for partial hydrolysis of the pyrimidine nucleotides recommended by Smith & Markham (1950).

In the experiment shown in Table 4, the modification, method (*d*), of the ionophoresis procedure was used in which ultraviolet absorption measurements were made on the eluates instead of using the phosphorus estimations employed by Davidson & Smellie (1952*a*). This yielded results essentially the same as those provided by method (*c*) and had the advantage of being suitable for smaller quantities of material than the original ionophoretic method.

Our results are expressed in terms of molar ratios relative to adenine. It was therefore necessary to determine whether the low figure for uracil obtained by method (*a*) was in fact related to the uracil itself rather than to the adenine or to both.

The low values for uracil obtained by method (*a*) appeared to be related to the method of hydrolysis rather than to the method of separation of the hydrolysis products and were obtained for any hydrolysis time between 15 and 360 min. (Table 5).

When yeast RNA was hydrolysed with perchloric acid and the base ratios calculated from the extinction of the paper eluates at the absorption maximum only (Wyatt, 1951), it was observed that the cytosine/adenine ratio was the same as was obtained by the difference method, whereas the uracil/adenine ratio was higher (Table 5).

It was also noted that the material eluted from the uracil spot on chromatograms of perchloric acid hydrolysates of RNA or nucleotide mixtures gave values for the ratio E_{259}/E_{280} in the range 2·0–4·8 rather than the expected 6. This anomaly was not observed when digests of pure bases were used.

It was clear therefore that uracil obtained after perchloric acid digestion of RNA was contaminated. Since the observed R_F value of cytidylic acid (0·76) is close to that of uracil (0·73) it seemed likely that the contamination might be due to traces of unhydrolysed cytidylic acid. Consequently, the material eluted from the uracil spot would give a

relatively high optical density at 280 mμ. since the absorption maximum of cytidylic acid is 278 mμ. The apparent value for uracil would therefore be low owing to reduction of the Δ value.

To test this possibility a sample of radioactive cytidylic acid was prepared by ionophoresis of an alkaline digest of RNA from the liver of a rat which had received ^{32}P. The nucleotide was digested with perchloric acid and subjected to chromatography as described in method (*a*). The chromatogram was cut up into strips for elution and the eluates examined for optical density at 275 mμ. and for radioactivity.

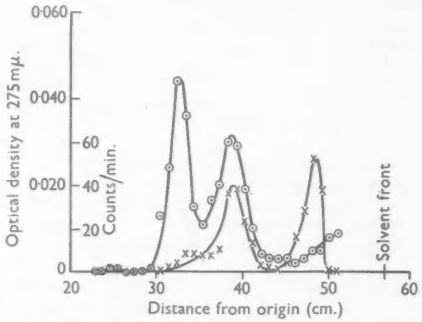

Fig. 1. Optical density, ⊙—⊙, and radioactivity (counts/min.), ×—×, of eluates from 1 cm. strips of a chromatogram of a perchloric acid digest of [^{32}P]cytidylic acid. The optical density peak at 31 cm. corresponds to cytosine and the radioactivity peak at 48 cm. to inorganic phosphate. The two peaks at 39 cm. indicate the presence of a component which exhibits the characteristic ultraviolet absorption spectrum of cytidylic acid.

Cytosine was found in the expected position on the chromatogram while inorganic phosphate was detected near the solvent front using the spray reagent of Hanes & Isherwood (1949). In addition, however, that portion of the chromatogram corresponding to the R_f value of uracil contained some unchanged cytidylic acid as shown by radioactivity and optical density measurements (Fig. 1).

In view of these results it was decided to concentrate on method (*d*) (alkaline hydrolysis followed by ionophoresis of the nucleotides) as being the most reliable available. It was applied to the RNA found in the cytoplasmic components and nuclei of rat liver with the results shown in Table 6. It is clear that there is no important difference between the RNA's from the three cytoplasmic sources. All show the same pattern of high proportions of guanine and cytosine. Such a pattern is also shown in the specimen of whole rat-liver RNA mentioned in Table 3 and distinguishes liver RNA sharply from yeast RNA in which the bases are present in almost equimolecular proportions (Table 4).

The method of pentose identification was applied to the RNA's from all four morphological components of rat-liver tissue. In each case the sugar was found to be chromatographically identical with ribose. Since ribose is easily distinguished from the other pentose by its high R_g value in the solvent system employed, there can be no doubt that the sugar present in all four liver RNA fractions is ribose. This has been confirmed for RNA from whole liver cytoplasm using the butanol-ethanol-water solvent system of Chargaff *et al.* (1950).

Table 7 shows the molar composition of RNA from maternal and foetal rabbit liver. Again the three cytoplasmic fractions show the same pattern and are apparently identical. Rabbit-liver RNA, like rat-liver RNA, is characterized by high relative proportions of guanine and cytosine, and appears to be the same in the foetus as in the mother.

Figures for the cytoplasmic fractions of regenerating rat liver 26 hr. after partial hepatectomy are also given in Table 7. At this time the turnover of the RNA appears to be near its maximum (Johnson & Albert, 1952; Eliasson *et al.* 1951), but there appears to be no significant change in the relative molar proportions of bases.

The slight difference in composition between the rabbit-liver RNA shown in Table 3 and the pregnant rabbit-liver RNA in Table 7 suggests that liver RNA might vary in composition from one animal to another. This view is supported by the results of analysis of specimens of liver RNA from four different rabbits in which the molar proportions of guanine to adenine as 10 were 19·7, 17·0, 16·5 and 15·4. This divergence is not due to experimental error in the method of analysis since agreement among replicate analyses of the same specimen is satisfactory (Table 4). The composition of rat-liver RNA, on the other hand, appears in our experience to vary only within very narrow limits.

Table 6. *Analyses of ribonucleic acid from different morphological fractions of a rat liver, expressed as molar ratios relative to adenine as* 10

Analysis by method (*d*).

	Mitochondria	Microsomes	Cell sap	Nuclei
Adenine	10	10	10	10
Guanine	16·9	16·9	16·9	9·0
Cytosine	15·1	14·7	14·6	11·5
Uracil	11·0	10·3	11·3	11·1
Purine/pyrimidine ratio	1·03	1·08	1·04	0·84

Table 7. *Analyses of ribonucleic acid from different morphological fractions of pregnant and foetal rabbit-liver tissue and regenerating rat-liver tissue expressed as molar ratios relative to adenine as* 10

	Mitochondria	Microsomes	Cell sap	Whole cytoplasm
		(*a*) Pregnant-rabbit liver		
Adenine	10	10	10	10
Guanine	15·9	15·6	15·4	15·8
Cytosine	15·6	15·4	15·3	15·2
Uracil	10·3	10·2	10·2	11·1
		(*b*) Foetal rabbit liver		
Adenine	10	10	10	10
Guanine	15·4	16·1	15·4	15·4
Cytosine	15·5	15·5	15·3	15·6
Uracil	10·4	10·4	9·8	10·1
		(*c*) Regenerating rat liver		
Adenine	10	10	10	10
Guanine	17·0	16·7	17·2	16·6
Cytosine	14·2	15·1	14·7	14·1
Uracil	10·3	10·4	10·5	10·2

DISCUSSION

In the analysis of RNA the method of preliminary hydrolysis is of prime importance. Hydrolysis with N-hydrochloric acid produces purine bases and pyrimidine nucleotides, but the latter are partially degraded so that a correction has to be applied (Smith & Markham, 1950). Perchloric acid hydrolysis on the other hand yields all four bases, but it is clear from our results that cytidylic acid is not quantitatively hydrolysed under the conditions employed (Fig. 1), with the result that the figures for both cytosine and uracil are unreliable. No improvement results from an increase in the hydrolysis time (Table 5). Vischer & Chargaff (1948*b*) have similarly reported resistance to hydrolysis by formic acid.

These difficulties can be overcome by the use of alkaline hydrolysis provided that the conditions are such as to avoid deamination of cytidylic acid (Davidson & Smellie, 1952*a*). Although a mixture of four nucleotides cannot be completely separated by the paper chromatographic methods of Carter (1950), of Magasanik, Vischer, Doniger, Elson & Chargaff (1950), and of Smith & Markham (1950), adequate separation is easily achieved by ionophoresis on paper which can be adapted to the analysis of very small amounts of material by the use of method (*d*) described above. This is in our opinion the most satisfactory method for the analysis of RNA.

Wyatt (1951) has commented on the low percentage of the total phosphorus of yeast RNA accounted for by calculation from the amounts of bases recovered from chromatograms of perchloric acid digests. We have confirmed this observation; in one experiment, for example, the recovery on this basis was 86 % whereas the corresponding recovery after separation of the nucleotides by the ionophoretic method (*d*) was 97 % (Table 4).

It is clear from the results in Table 6 that there is no essential difference in composition of the RNA's from the three cytoplasmic sources in rat or rabbit liver. In a brief note, Elson & Chargaff (1951) mention the similarity in composition of the RNA's from mitochondria and submicroscopic particles of rat liver without quoting figures. Both were characterized by high molar proportions of guanine and cytosine such as we have recorded in Table 6. This general pattern has been observed for whole rat- and rabbit-liver RNA by Volkin & Carter (1951) and for the liver tissue of other species by Chargaff *et al.* (1950).

Although nuclear RNA contains the same ribonucleotides as cytoplasmic RNA (McIndoe & Davidson, 1952) it has been found that the proportions may vary widely from animal to animal and are much more readily affected by the conditions of hydrolysis than is the case with cytoplasmic RNA. While relatively low guanine and cytosine values occur in the sample of nuclear RNA shown in Table 6, higher values have been found in many batches of nuclei (McIndoe, unpublished results) although a lower guanine value than is commonly found in cytoplasmic RNA is recorded in the few results available in the literature for the nuclear RNA of rat and calf liver (Elson & Chargaff, 1951; McIndoe & Davidson, 1952; Marshak, 1951). Nuclear RNA can also be distinguished from cytoplasmic RNA by its much more rapid uptake of ^{32}P (Marshak & Calvet, 1949; Barnum & Huseby, 1950; Jeener & Szafarz, 1950; Davidson, McIndoe & Smellie, 1951; Smellie *et al.* 1953) and of isotopically labelled orotic acid (Potter, Recknagel & Hurlbert, 1951) and of [^{14}C]formate (Smellie & McIndoe, 1952).

The results of our chromatographic experiments make it clear that the same pentose is found in all three cytoplasmic RNA's and in nuclear RNA, and that it is chromatographically identical with ribose. The pentose of whole liver RNA from pig, sheep, calf and man has been shown to be chromatographically identical with ribose by Chargaff *et al.* (1950). Since the pentose in the whole liver RNA of the sheep has been conclusively proved to be ribose after identification of the *p*-bromophenylhydrazone by Davidson & Waymouth (1944) there would appear to be little doubt that the sugar in liver RNA, from whatever part of the cell, is in fact ribose. There is therefore every justification for using the term ribonucleic acid rather than pentosenucleic acid when referring to liver tissue.

SUMMARY

1. Three methods for the determination of the proportions of bases in ribonucleic acid (RNA) have been compared; paper-chromatographic separation after hydrolysis with perchloric acid and after hydrolysis with N-HCl, and separation by ionophoresis on paper of the nucleotides obtained by alkaline hydrolysis. It is concluded that the last method is the most satisfactory and a modification for use with small amounts of material is described.

2. The RNA's from mitochondria, microsomes and cell sap of rat liver have been shown to have the same composition in terms of molar proportions of bases, and differ in composition from the RNA of the rat-liver nucleus. The molar proportions of bases in the RNA's from the cytoplasmic fractions of maternal and foetal rabbit liver or of normal and regenerating rat liver have been found to be the same.

3. The pentose sugar in the RNA's from rat-liver mitochondria, microsomes, cell sap and nuclei has been chromatographically identified as ribose.

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The Biochemistry of the Nitrifying Organisms

3. COMPOSITION OF *NITROSOMONAS*

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In recent years a number of investigations have been carried out on the composition of proteins, polysaccharides and whole cells of micro-organisms. Mazur & Clarke (1942) examined protein hydrolysates of a number of algae. Camien, Salle & Dunn (1945) investigated *Escherichia coli* and four strains of Lactobacilli and reviewed earlier work on this subject. Figures for amino-acids for various bacteria and yeasts are given by Stokes & Gunness (1946) and Freeland & Gale (1947). Recently, Fowden (1952) published the results of an analysis of the bulk proteins of *Chlorella vulgaris*. Bell & Northcote (1950) found that the cell-wall polysaccharides of baker's yeast consisted only of glucose. Work (1950) isolated a hitherto unknown amino-acid, αε-diaminopimelic acid, from *Corynebacterium diphtheriae*.

No information, however, is available on the composition of chemosynthetic organisms. These organisms are of special interest as they derive their energy entirely from simple inorganic reactions and use carbon dioxide or inorganic carbonates as their only source of carbon.

This paper gives the results of an investigation on the composition of *Nitrosomonas*, the chemosynthetic organism oxidizing ammonium ions to nitrite ions.

MATERIALS AND METHODS

Nitrosomonas was grown as described by Lees (1952) in 400 ml. medium.

Preparation of organisms for analysis. When the amount of nitrite formed in a culture vessel had risen to about 300 μg. NO_2-N/ml., corresponding to about 10 mg. organic carbon in the whole culture (Hofman & Lees, 1952), the culture was centrifuged and the insoluble inorganic salts with the adhering organisms were collected. After several washings with water the residues were shaken with about 150 ml. 5% acetic acid to dissolve the inorganic salts. The suspension was filtered through a very coarse sintered-glass filter to remove any large particles remaining and the filtrate was again centrifuged and the residue washed several times with small quantities of water.

Amino-acid analysis. The organisms were hydrolysed for 18 hr. with 6N-HCl at 110° in a sealed tube under an atmosphere of N_2. Excess HCl was removed by evaporation and the residue taken up in 1 ml. of water.

Nitrogen determinations. Total N was estimated in the hydrolysate by the micro-Kjeldahl method of Chibnall, Rees & Williams (1943); α-amino N was determined by the method of Pope & Stevens (1939); free NH_3-N estimations were carried out in the usual way on a Markham micro-distillation apparatus (Markham, 1942).

Amino-acid estimations. These were carried out essentially according to the method described by Fowden (1951). It was found, however, that under the conditions of our experiments more constant results could be obtained when control chromatograms of amino-acid solutions of known composition were run simultaneously with the mixtures of amino-acids to be analysed. These control solutions were of approximately the same composition as the unknown. A preliminary estimate of the composition of the unknown was obtained by following exactly the procedure as described by Fowden (1951). The amount of each amino-acid in the *Nitrosomonas* hydrolysate was then determined colorimetrically on a Unicam diffraction grating spectrophotometer at a wavelength of 570 mμ. using the corresponding amino-acid from the control chromatograms as a standard. Proline was estimated at 440 mμ.

Most of the amino-acids present in the hydrolysates could be separated on two-dimensional chromatograms (Consden, Gordon & Martin, 1944) using phenol-water 4:1 (v/v) in the presence of 0·1% ammonia as the first, and butanol-acetic acid-water (Partridge, 1948) as the second, solvent.

Leucine, isoleucine, methionine and phenylalanine did not appear as separate spots under these conditions. They were separated by using water-saturated *tert.*-amyl alcohol (Work, 1949) on a one-dimensional chromatogram and developing for 4 days in the presence of diethylamine vapour. In agreement with Fowden (1952) it was found that phenylalanine did not occupy the same relative position in this solvent as that described by Work (1949), but appeared between methionine and isoleucine.

Sugar analysis. For the qualitative analysis of the sugar components about 12 mg. of organisms were hydrolysed with 2 ml. N-H_2SO_4 for 3 hr. at 100°. The insoluble cell fragments were filtered off and the H_2SO_4 removed as $BaSO_4$ or by treating the hydrolysate with an anion-exchange resin (Amberlite *IR-4B*) in a column of 0·7 × 10 cm. The resin was washed first with N-HCl for 2 hr., then with N-NH_3 solution for 2 hr. and finally with distilled water until the reaction of the effluent was neutral. The filtered hydrolysate (about 4 ml.) was added to the column and washed down with 5 ml. of water. The first 2 ml. of effluent were discarded. The next 5 ml. (about pH 6·0) were collected,

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Table 1. *Amino-acid composition of hydrolysates of whole cells of* Nitrosomonas *in comparison with* Corynebacterium diphtheriae, Chlorella vulgaris *and brewer's yeast*

	Amino-acid N (as % total N)			
Amino-acid	*Nitrosomonas*	*C. diphth.* (Work, 1949)	*Chlorella* (Fowden, 1952)	*Yeast* (Lindan & Work, 1951)
Aspartic acid	5	4	6·4	8
Glutamic acid	8	8	7·8	10
Serine	2	2	3·3	4
Glycine	8	5	6·2	8
Threonine	4	1	2·9	7
Alanine	8	8	7·7	10
Tyrosine	1	2	2·8	2
Leucine	3	4	6·1	6
Isoleucine	4	4	3·5	4
Valine	7	7	5·5	8
Histidine	3	6	3·3	8
Arginine	8	20	15·8	10
Lysine	8	5	10·2	9
Phenylalanine	4	3	2·8	1·6
Methionine	2	3	1·4	0·5
Proline	4	5	7·2	3
Cystine	—	—	0·2	0·9
Typtophan	—	—	2·1	0
Ammonia N	8	—	6·1	—
Total N	87	87	101·3	100

—, Indicates that no estimation has been made.

concentrated *in vacuo*, and the residue, taken up in water (0·2 ml.), was used for analysis.

Chromatography. For one-dimensional paper partition chromatograms, 20–40 μl. of hydrolysate were used and the following solvents: butanol-acetic acid-water (Partridge, 1948), butanol-pyridine-water, 3:1:1, and butanol-ethanol-water, 4:1·1:1·9 (Hough, Jones & Wadman, 1950). Two-dimensional chromatograms were obtained using phenol-water, 4:1, as the first and the butanol-acetic acid-water mixture as the second developing agent. *p*-Anisidine hydrochloride (3%, w/v, in butanol) was used as a spray reagent (Hough *et al.* 1950).

RESULTS AND DISCUSSION

Table 1 shows the average values obtained from six determinations. Under the conditions used in these experiments it was not found possible to reduce the probable error of the individual values below about 10%. For this reason all the values are given to the nearest whole figure.

It can be seen from the table that the distribution of the amino-acids is similar to that obtained for *Corynebacterium diphtheriae* (Work, 1949) and for *Chlorella vulgaris* (Fowden, 1952) and for brewer's yeast (Lindan & Work, 1951). The content of total basic amino-acids and of tyrosine is lower than in the other three organisms.

The presence of tryptophan has been confirmed by paper chromatography of a barium hydroxide hydrolysate using *p*-dimethylaminobenzaldehyde for spraying. Cystine was present in small amounts only. No attempts have been made to estimate either tryptophan or cystine. $\alpha\epsilon$-Diaminopimelic acid, which was found to be part of a great number of micro-organisms (Work & Dewey, 1952) but does not seem to occur in material of non-bacterial origin, was not detected in our hydrolysates.

Attempts were made to extract and estimate free amino-acids from the cells. For this purpose the organisms were extracted repeatedly with 95% ethanol, prior to treatment with acetic acid. The pooled extracts after they had been reduced to a very small volume did not contain any detectable amount of amino-acids.

Table 2. *Sugar composition of hydrolysates of* Nitrosomonas

(Detected on paper chromatograms after spraying with 3% *p*-anisidine-HCl in *n*-butanol.)

	Intensity of spot	
	In visible light	In ultraviolet light
Galactose	+ + +	+ + +
Ribose	+ + +	+ + +
Rhamnose	+ +	+ +
Xylose	+	?
Unknown	+	+ + +

+ + +, strong; + +, moderate; +, weak, ?, doubtful.

In Table 2 results of a paper-chromatographic analysis of the sugar components are presented. One-dimensional chromatograms were run with the mixture of the unknown and with known sugars on the same sheet. On two-dimensional chromatograms the unknown mixture and known sugars as markers were run together. When the spots of the

unknown and the marker appeared as one single spot it was assumed that the unknown sugar was identical with the marker. On all chromatograms run with four different solvent mixtures there appeared an unidentified spot which reacted with *p*-anisidine hydrochloride to give a pale yellow colour and a fairly strong bluish white fluorescence in the ultraviolet light. Its R_g value of 0·05–0·11 in all four solvents tested was different from that of a series of known hexoses and pentoses.

It is of interest to find that the sugars, galactose, rhamnose and xylose, which were present in the hydrolysates of *Nitrosomonas* were also found by Hough, Jones & Wadman (1952) in hydrolysates of carbohydrates extracted from the freshwater alga *Nostoc*. These carbohydrates contain in addition glucose, which could not be detected in our hydrolysates, uronic acids, and an unidentified component. From the information available it is not possible to obtain any indication as to whether the unidentified component in the *Nitrosomonas* and *Nostoc* hydrolysates could be the same.

SUMMARY

1. Hydrolysates of whole cells of *Nitrosomonas* have been investigated for amino-acids and sugars.

2. The results of a quantitative study of the amino-acid composition were compared with figures for *Corynebacterium diphtheriae* and *Chlorella vulgaris*.

3. Four sugars were detected on paper chromatograms. Their chromatographic properties were identical with those of galactose, ribose, rhamnose and xylose. In addition an unidentified spot was found.

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A Radiochemical Method for the Micro-estimation of α-Amino-Acids Separated on Paper Partition Chromatograms

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Consden, Gordon & Martin (1944) successfully separated mixtures of amino-acids on paper partition chromatograms and thereby provided a sensitive method for the qualitative analysis of protein hydrolysates. Although the amounts of amino-acids thus separated were only of the order of micrograms, the elegance of the technique stimulated many workers to attempt their quantitative estimation. Bull, Hahn & Baptist (1949) used a standard method for the colouring of amino-acid spots on paper chromatograms with ninhydrin, and estimated their amino-acid contents by scanning them with a photometer. The differences in the size of amino-acid spots on chromatograms formed the basis of the method used by Fisher, Parsons & Morrison (1948), who showed that the area of a spot was linearly related to the logarithm of its amino-acid content. Between pH 8 and 12 cupric ion will react with α-amino-acids to give complexes of the type $(H_2N.CHR.COO)_2Cu$. This reaction was employed by Martin & Mittelmann (1948) and Woiwod (1949), who used copper phosphate suspensions at pH 9 to form the soluble copper complexes of amino-acids; the former authors estimated

the copper in the complexes polarographically and the latter by a colorimetric method. Both these methods are essentially modifications of that used by Pope & Stevens (1939).

When radioactive isotopes became generally available, they were applied to the microanalysis of amino-acid mixtures. Two such radiochemical methods have already been described. Keston, Udenfriend & Levy (1947, 1950) converted amino-acids into their *p*-iodobenzenesulphonyl derivatives which were labelled with ^{35}S or ^{131}I or with both. The radioactive derivatives were separated on paper chromatograms and the amino-acids estimated by scanning the spots systematically with a Geiger-Müller counter. Wieland, Schmeiser, Fischer & Maier-Leibnitz (1949), after separating amino-acids in the conventional way on chromatograms, allowed a solution of copper acetonylacetate containing ^{64}Cu slowly to ascend the paper, thereby forming radioactive copper-amino-acid complexes *in situ*. The amino-acids were then estimated by scanning the paper with a Geiger-Müller counter.

We now describe a method similar to that of Martin & Mittelmann (1948), with the important modification that the copper phosphate suspension was prepared from cupric chloride containing ^{64}Cu. The results obtained in applying the method to the analysis of salmine sulphate are also described.

EXPERIMENTAL

On beginning this work we envisaged a simple modification of the method of Woiwod (1949) by incorporating ^{64}Cu into the copper phosphate suspension, and estimating the amount of Cu in the soluble copper complexes of amino-acids radiometrically. However, when we prepared the copper phosphate suspension according to Woiwod's directions, we were unable to reproduce the *F* ratios, of weight of α-amino N to weight of Cu reacting, which he obtained for various amino-acids. Hence we experimented with several methods of precipitating copper phosphate, and suspending it in different buffer systems, finally adopting the procedure described below, which in our hands has given the most satisfactory results. Even so, differences in properties of phosphate suspensions were noted. The average values of the *F* ratio for glycine, alanine, valine, serine, histidine and aspartic acid, which we obtained using phosphate suspensions prepared by the same procedure but at different periods, were in accord with those given by Woiwod (1948). However, the deviations from the average value for any particular amino-acid were between 5 and 8%. These variations can only be attributed to variations in the phosphate suspensions, and may be due to differences in grain size. Since the half-life of ^{64}Cu is short (12·8 hr.), it was necessary to prepare fresh suspensions weekly, and we were unable to use them for more than 3 days. Thus we were restricted to a small number of determinations with each suspension. In early work on the determination of amino-acids in spots cut from one-dimensional paper partition chromatograms, we used the average *F* ratios for amino-acids found by Woiwod and by us, but the results were inconsistent, recoveries being approximately 100±8%. This was to be expected in view of the inconsistency of the *F* ratio previously described. Considering the small amounts of amino-acid estimated (1–10 μg. of α-amino N), the accuracy obtained was reasonable, but insufficient for our purposes. The procedure was abandoned in favour of the one outlined below, in which any slight variations between copper phosphate suspensions are eliminated.

METHOD

Preparation of copper phosphate suspension containing ^{64}Cu. 0·5 g. of Cu foil, which had been irradiated at the Atomic Energy Establishment, Harwell, was dissolved in the minimum of 5 N-HNO_3, 10 ml. of 10 N-HCl were added and the solution evaporated to dryness. A further 5 ml. of HCl were added, the solution again evaporated, and the dry chloride dissolved in 50 ml. of distilled water. The resultant solution contained about 10 mc. of ^{64}Cu.

10 ml. of the radioactive $CuCl_2$ solution, and a solution of 1·1 g. of $Na_2HPO_4 . 12H_2O$ in 15 ml. of distilled water, were run simultaneously from two burettes at controlled rates into 150 ml. of stirred distilled water, so that at no time was there a large excess of cupric ion over phosphate ion. The pH was adjusted to 9 by the addition of 0·1 N-NaOH and the copper phosphate precipitate centrifuged off, washed with 2% (w/v) aqueous $Na_2B_4O_7 . 10H_2O$ containing 17·5 g. of $Na_2HPO_4 . 12H_2O$/l. and suspended in 200 ml. of this solution. The suspension was allowed to 'age' overnight before use.

Chromatographic technique and method of estimation. The amino-acids were separated on one-dimensional chromatograms. A sheet of Whatman no. 4 or no. 3 MM paper (22 × 22 in.) was divided by thin pencilled lines into eleven serially numbered strips of equal width; measured samples of the solution of amino-acids to be analysed were applied to strips 8, 10 and 11 by means of a capillary micropipette. Progressively increasing amounts of a control mixture containing the same amino-acids in approximately the same proportions as those in the mixture under examination were applied to strips 1, 2, 4, 5 and 7. Known amounts of the same control mixture were applied to strips 3, 6 and 9 in approximately the same amount as on strip 7. In all cases the solutions were applied as circular spots in the centres of the strips, along a line drawn 7 cm. from one edge of the paper. After development the chromatogram was dried and strips 3, 6 and 9 were cut out, sprayed with ninhydrin and heated to reveal the positions of the amino-acid spots. The strips were then re-aligned with the remainder of the chromatogram, and their amino-acid spots used as guides to cutting the spots on the other strips. Care was taken that the paper cuts carrying the spots for any one amino-acid should all have the same area.

The paper cuts were divided into thin strips, dropped into stoppered Pyrex test tubes, and 2 ml. of 0·18 M-Na_2HPO_4 solution added to each tube. After standing overnight, 2 ml. of the radioactive copper phosphate suspension were added to each tube, and they were shaken intermittently over a period of 30 min. The mixtures were filtered through pleated Whatman no. 42 papers and a 1 ml. sample removed from each filtrate. These were counted in a polythene dish beneath a Geiger-Müller counter, and the counts corrected for the decay of ^{64}Cu. Standard curves were then plotted for each amino-acid, showing the amount as μg. of α-amino N

against the corresponding counts/min. The amino-acid contents of the mixture under analysis were then read off by reference to these curves.

Radiochemical assay of ^{64}Cu. In preliminary experiments, 2 ml. of the radioactive filtrates containing the copper-amino-acid complexes were diluted to 10 ml. with distilled water and counted in a Twentieth-Century Electronics Type M. 6 liquid counter. We found that there were several drawbacks to this counting method. The window thickness of the counter is of the order of 35 mg./cm.^{2} which appreciably reduces the β-particle counts from ^{64}Cu. Moreover, after every count with an active sample, and subsequent rinsing of the counter with distilled water, the background was invariably slightly higher than before. This 'memory effect' due to adsorption of Cu on the glass walls could be effectively removed by cleaning the counter with HCl, but the time involved in this procedure reduced that available for counting. We next prepared solid samples by evaporating 0·2 ml. portions of the filtrates to dryness in aluminium trays under a radiant heater, and counted them beneath the window of a G.E.C. Type G.M. 4 counter. The trays were 20 mm. in diameter and 2 mm. deep, and were pressed out from 26-gauge aluminium sheet, which is sufficiently thick to give saturation back-scattering for the β-particles from ^{64}Cu (Yaffe & Justus, 1949). Inspection of the samples prepared in this way showed that they were far from uniform in thickness, most of the deposite being concentrated around the tray periphery, but all the counts obtained lay on the calibration curve within ±3%, when counted to ±1% statistical counting error. The most satisfactory method of assaying ^{64}Cu in our experience, however, is to pipette 1 ml. of radioactive solution into a polythene dish and count it beneath an end-window counter of the G.E.C. G.M. 4 or E.M.M. 2 types. The polythene dishes we used were turned from 1 in. diameter polythene rod, and had internal diameter 23 mm. and depth 7 mm. We tested this method fairly rigorously by counting twenty 1 ml. samples of a ^{64}Cu solution successively in the same polythene dish; the counts obtained showed no variations other than those to be expected from a Poisson distribution. The polythene dishes retain traces of ^{64}Cu, but this can be removed by a brief wash in an acid $CuCl_2$ solution followed by a distilled-water rinse. The method is recommended for the routine radiometric assay of liquid samples.

RESULTS

The results showed a linear relationship between the amounts of amino-acid and the counts given by the ^{64}Cu in the corresponding copper-amino-acid complexes. Fig. 1 shows this relationship plotted for DL-alanine. Similar curves were obtained for glycine, DL-valine, DL-isoleucine, L-leucine, DL-proline, DL-serine and L-arginine, and for L-cysteic, L-aspartic and L-glutamic acids. In general, the amounts of amino-acids used were in the range 0–12 μg. of α-amino N, but no significant deviation from linearity has been found for the range 0–50 μg. of α-amino N.

The estimation of isoleucine, valine and arginine in salmine sulphate

Analyses of isoleucine and valine in a mixture of amino-acids made up to simulate a hydrolysate of salmine sulphate. As a preliminary to the estimation of isoleucine and valine in a hydrolysate of salmine sulphate the percentage recoveries of these amino-acids from a mixture of the constituent amino-acids of salmine, in the approximate proportions in which they occur in the protamine, were determined. A mixture of the seven amino-acids, DL-isoleucine, DL-valine, DL-alanine, glycine, DL-proline, DL-serine and L-arginine in the molecular ratios of 1:3·15:1:4:6:6:50 was prepared, and run on one-dimensional paper chromatograms against a mixture of DL-isoleucine and DL-valine in the molecular ratio of 1:3·15 using collidine as developing solvent. Whatman no. 3 MM paper was used in preference to Whatman no. 4, since the

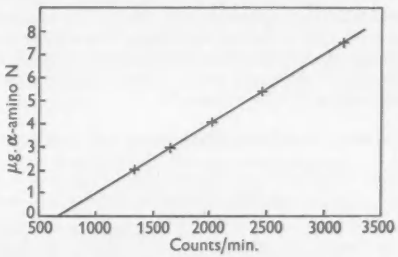

Fig. 1. Curve showing the counts given by the copper complex of increasing amounts of alanine run on a one-dimensional butanol chromatogram.

former gives compact spots with much heavier amino-acid loads than the latter; even so the amounts of isoleucine which could be satisfactorily separated were only of the order of 1·5 μg. of α-amino N. This amount corresponds to a total of 1·2 mg. of amino-acid mixture/spot. The results of eight analyses of isoleucine and valine appear in Table 1. It will be noted that all the isoleucine recoveries with one exception lie within the range 100±3% and all the valine recoveries within the range 100±2%.

Table 1. *Estimations of* DL-*isoleucine and* DL-*valine separated from a mixture of* DL-*isoleucine,* DL-*valine, glycine,* DL-*alanine,* DL-*proline,* DL-*serine and* L-*arginine*

(For details see text.)

	μg. N			
	Isoleucine		Valine	
Sample no.	Applied	Found	Applied	Found
1	1·40	1·38	4·48	4·48
2	1·53	1·47	4·90	5·00
3	1·55	1·57	4·96	4·95
4	1·51	1·48	4·83	4·92
5	1·50	1·44	4·80	4·90
6	1·50	1·54	4·80	4·88
7	1·21	1·24	3·89	3·81
8	2·06	2·10	6·60	6·55

Analyses of isoleucine and valine in salmine sulphate hydrolysates. The isoleucine and valine contents of two hydrolysates of salmine sulphate were determined. The first hydrolysate was prepared by refluxing 1·351 g. of dry salmine sulphate in 20 ml. of 5N-HCl for 24 hr.; in the second, 1·425 g. of dry salmine sulphate with 28·1 mg. added DL-isoleucine were treated in the same way. The

salmine sulphate used for these hydrolysates was air-dry and its moisture content was determined by drying a previously weighed portion to constant weight in an oven at 102°. The isoleucine added to the second hydrolysate was sufficient approximately to treble the isoleucine content. Five analyses of each of the amino-acids were made on each hydrolysate, the separations being carried out on Whatman no. 4 one-dimensional paper chromatograms run in collidine. A mixture of isoleucine and valine in the molecular ratio 1:3·15 was used to plot the standard curves. The SO_4^{2-} content of the salmine sulphate used was estimated gravimetrically as $BaSO_4$ and found to be 19·9%, and its total N content estimated (Kjeldahl) to be 24·2%. By employing a factor of 0·797 to convert the weight of salmine sulphate to salmine, the isoleucine and valine contents of the hydrolysate were determined as g. of amino-acid/100 ml. of salmine, and are given in Table 2.

Table 2. *Analyses of isoleucine and valine in two salmine sulphate hydrolysates*

	g./100 ml. salmine (free base)			
	Hydrolysate of salmine sulphate alone		Hydrolysate of salmine sulphate with added isoleucine*	
Sample no.	Isoleucine	Valine	Isoleucine	Valine
1	1·30	3·70	3·86	3·70
2	1·29	3·58	3·91	3·70
3	1·26	3·57	3·85	3·64
4	1·26	3·72	3·84	3·65
5	1·25	3·60	3·88	3·68

* 2·60 g. isoleucine/100 g. salmine (free base).

From Table 2 the isoleucine content of the normal salmine sulphate hydrolysate is 1·27±0·01 g. and the valine content 3·65±0·03 g./100 g. of salmine, where the errors are the standard errors of the mean. The isoleucine and valine contents of the hydrolysate, to which isoleucine was added before hydrolysis, are 3·87±0·01 g. and 3·70±0·01 g./100 g. of salmine respectively. Since the amount of isoleucine added was equivalent to 2·60 g./100 g. salmine, it is evident that the recovery of isoleucine from the hydrolysate is 100%.

Estimation of arginine in salmine sulphate hydrolysate. The normal salmine sulphate hydrolysate (i.e. with no added isoleucine) used above was diluted 1:10 with water, the resultant solution applied to Whatman no. 4 paper with the necessary number of arginine standard spots; the chromatograms were developed in collidine. The average of ten analyses for arginine was 85·4±0·2 g. of arginine/100 g. of salmine, the highest value determined being 87·8 g. and the lowest 84·6 g./100 g. of salmine respectively.

Losses of amino-acid during paper chromatography

The possible losses that occur during the chromatography of amino-acids were investigated as follows. Known amounts of an amino-acid were run on a one-dimensional chromatogram. To certain of the strips, however, no amino-acid was applied before running, but paper cuts were made in the usual way. Known amounts of amino-acid were now added, converted into the copper complex, and counted. By reference to a standard curve drawn from these results the percentage 'recovery' of amino-acid which had been run on the chromatogram could be calculated: for collidine it was 100±3%, for phenol 98±3%, for *n*-butanol 100±3%.

There has been considerable discussion as to the extent of the loss of amino-acid that occurs during paper chromatography, the principal sources of loss being attributed to (*a*) loss during development of the chromatogram (Woiwod, 1949), and (*b*) loss due to partial decomposition of amino-acid during drying of the chromatogram (Fowden, 1951; Brush, Boutwell, Barton & Heidelberger, 1951; Novellie, 1950). Our results are incompatible with the occurrence of appreciable losses of type (*a*). It is reasonable to suppose that the magnitude of losses of type (*b*) will depend not only on the temperature at which drying is carried out, but also on the speed of drying, i.e. the time during which the amino-acid is in contact with hot solvent. This latter factor is greatly dependent on the velocity of the air current in which drying takes place. Our chromatograms were dried in a powerful blast of air which gradually attained a temperature of 100°, and under these conditions the solvent on the paper is effectively removed before high-temperature reaction with the amino-acid spots can occur.

DISCUSSION

The present method for the micro-estimation of amino-acids separated on one-dimensional paper chromatograms compares favourably in accuracy with other methods. In practice we found the accuracy to be ±3% on amounts of 1 μg. of α-amino N and ±2% for amounts of 2 μg. of α-amino N and above. This degree of precision is not increased if amounts up to 50 μg. of α-amino N are used. However, the accuracy attainable is better than has been given by any other method employing paper partition chromatograms to separate amino-acids and the method is more sensitive than most of those hitherto described. Furthermore, the accuracy is comparable with that of other methods of amino-acid analysis, i.e. Stein & Moore (1948) claim recoveries of 100±3% for the amino-acids which they separate by their starch column chromatographic technique.

The method has the advantage that large numbers of accurate analyses can be carried out in a relatively short time, for, although the half-life of ^{64}Cu is only 12·8 hr. we have been able to use the radioactive copper phosphate suspensions over a period of 2 days, during which about thirty-six analyses were made. The principal limitation of the method in its application to one-dimensional paper chromatograms is the fact that, although spraying with ninhydrin may clearly distinguish between two amino-acid spots, the separation between them may not be sufficient for clean cuts to be made solely by reference to a guide strip.

Isoleucine, valine and arginine are the only amino-acids of the seven present in hydrolysates of salmine sulphate which we have separated sufficiently well

Table 3. *Amounts of isoleucine, valine and arginine present in salmine*

	Amino-acid (g./100 g. of salmine)						
				Velick & Udenfriend (1951)			
Amino-acid	Tristram (1947)	Hamer & Woodhouse (1949)	Block *et al.* (1949)	Microbiological assay*	Isotope dilution	Ion exchange	Present authors
Isoleucine	1·61	1·8	1·2	1·1	N.d.	N.d.	1·27
Valine	3·1	3·8	4·1	4·3	5·0	4·7	3·65
Arginine	85·2	87·2	N.d.	N.d.	N.d.	84·5	85·4

* Microbiological analyses by Dr G. B. Ramasarma.
N.d. = not determined.

on one-dimensional chromatograms for accurate determination. Our estimates of the amounts of these amino-acids present in salmine are shown in Table 3 together with the analyses of Tristram (1947), Hamer & Woodhouse (1949), Block, Bolling, Gershon & Sober (1949) and Velick & Udenfriend (1951). We find that isoleucine, valine and arginine are present in salmine in the ratios of 1:3:50 (the exact isoleucine to valine ratio from our figures being 1:3·22), and such ratios would require a somewhat higher molecular weight for salmine than the figure of 8000 calculated by Tristram (1947), who found the ratios of these amino-acids to be 1:2:40. The salmine sulphate we used was prepared by Boots Pure Drug Co. Ltd. from salmon milt obtained from Vancouver, British Columbia; it seems probable, therefore, that the salmon came from the Fraser River. Tristram (1949) used salmine sulphate prepared from Spring or Chum salmon.

A full analysis of salmine sulphate has been carried out in these laboratories, and the results of this analysis, together with a fuller discussion of the salmine problem, will form the subject-matter of another paper.

SUMMARY

1. A radiometric method is described for the micro-estimation of amino-acids separated on one-dimensional paper chromatograms. They are converted to their copper complexes with copper phosphate containing ^{64}Cu; 1 μg. of α-amino N can be estimated with an accuracy of ± 3 % and 2 μg. of α-amino N and above, with an accuracy of ± 2%.

2. Analyses of isoleucine, valine and arginine in salmine sulphate are reported.

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Tissue Silicon: A Study of the Ethanol-soluble Fraction, using ^{31}Si

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The view that silicon in animal tissues occurs in combination with organic groups has appeared repeatedly in the literature. As early as 1897 Drechsel & Winogradow described the extraction from goose feathers of an orthosilicate of the formula $Si(OC_{34}H_{59}O)_4$, stated to be an ester of a dihydric alcohol homologous with cholesterol. Isaacs (1924) inferred the presence of silicolipins in brain. Holzapfel (1943*a*), investigating the composition of lung tissue in silico-tuberculosis, found organosilicates, 'compounds in which the free valencies of the silica framework, instead of being satisfied with inorganic elements, such as calcium and magnesium, are satisfied with organic radicals'. She further reported both nitrogen-free and nitrogenous organic silicon compounds as derivatives of carbohydrates and proteins.

Holzapfel (1942) reported that silicon compounds could be extracted from silicotic lungs by alcohol and ether, and that Debye-Scherrer photographs of these extracts and of substances similarly extracted from goose feathers showed similarities, but she gave no experimental evidence of this. Holzapfel (1943*b*) found that ether removes only fats and cholesterol from blood, but that subsequent extraction with a mixture of ether and ethanol gives a fraction containing silicon, together with phosphatides, cholesteryl esters and soaps, and assumed that compounds similar to the phosphatides exist in which one or more phosphorus atoms are replaced by silicon. Assuming that the whole of the blood silicon is in organic combination, the insolubility of the greater portion was explained by the suggestion that compounds of polysilicic acids possess a very low solubility. Similar extraction of lung tissue showed that it behaved differently from blood (Holzapfel, 1947); the phosphatide fractions were free from silicon. As it was found in the ethereal extract containing fats and esters, Holzapfel suggested that silicon also occurred in combination with glycerol esters.

Ohlmeyer & Olpp (1944) assumed that esters of silicic acid are present in the tissues and compared the ethanol-ester soluble silicon levels in several tissues. They found no significant variation in the 'organic silicon', although the urine gave a high value for non-extractable (inorganic) silicon.

Although silicon has been reported as occurring in so many different types of organic compounds in biological material, there is no case recorded in which a compound has been isolated, and this work was undertaken in a further attempt to elucidate the problem. It soon became apparent that the usual chemical methods of analysis for the determination of silicon were inadequate to deal with the very small amounts present in the fractions. For this reason much of this investigation has been carried out with isotopically labelled silicon.

Preliminary work (Holt, Yates & Tomlin, 1951) had shown that radioactive silicon, injected as silicic acid, is absorbed and is distributed over the tissues generally. A more extensive survey has confirmed that there is no large accumulation in any organ except the kidney, although the more vascular tissues give rather higher values. The liver, being the largest vascular organ, was chosen as the source of material for this investigation into the nature of the silicon complexes.

Earlier workers have used ethanol or ether-ethanol mixtures to remove 'organic silicon' compounds from tissue. Ethanol also removes water and it is possible that a part of the silicon removed may be silicic acid which is associated with this water. For this reason tissues were usually dried by azeotropic distillation before extraction. As there is no evidence that ethanol is the most efficient solvent and particularly because it has been shown that ethyl silicate may be formed by some extraction procedures (Holt & Yates, 1950), other solvents have also been used.

Fractionation of the ethanol-soluble extracts by the usual methods of fractional distillation and solvent partition have been attempted with little success; indirect methods have therefore been used. Attempts made to form the complexes *in vitro* have indicated their probable nature, and have explained why the usual methods of fractionation are unsuccessful.

METHODS

Preparation of isotopically marked silicate solution. The silicate solution used for injection was prepared from pure fused silica which was powdered, sealed in a fused silica tube and placed in the Harwell pile (BEPO) for 12 hr. A weighed amount of irradiated material was fused with three times its weight of Na_2CO_3 in a platinum crucible and the melt was dissolved in water to prepare a solution containing 1·0 mg. SiO_2/ml. Injections were made intraperitoneally into adult rats in the quantities indicated.

Procedure for counting. A Geiger counter of the annular type (Veall, 1948) was used. Variations in density among solutions were too small to necessitate self-absorption corrections. The proportion of injected silicate reaching the tissues was determined by comparing counts made on solutions prepared from the tissues with counts on known dilutions of the injected solution.

Decay curves plotted from measurements of the radioactivity of one of the stock solutions, and of a portion of kidney removed from an animal after injection showed only the 170 min. half-life characteristic of ^{31}Si and proved that impurity activities were negligible.

On account of the short half-life of ^{31}Si all operations were performed, of necessity, as rapidly as possible. The activated sample was received within 45 min. of its removal from the pile and injections were made within a further 30 min.

Method of assay of total marked tissue silicon. The tissue was washed to remove unabsorbed silicate which might contaminate the surface, chopped, dried at 100°, ignited in a platinum crucible and fused with Na_2CO_3. The melt was dissolved in water and the whole or a suitable portion was transferred to the Geiger counter.

Method for the extraction and assay of ethanol-soluble silicon complexes. The tissue was washed, chopped and dried by the Dean & Stark (1920) procedure using benzene to produce an azeotrope with the tissue water. The dried tissue was refluxed with ethanol for several hours. A part of the extract was filtered, its volume measured and, when necessary, reduced by evaporation. This solution was transferred to a Geiger counter for assay as above. Any deviations from these procedures are mentioned.

EXPERIMENTAL AND RESULTS

Extraction of tissues of ^{31}Si-injected mice

Distribution of injected silicate in several tissues of the body. The radioactive solution was injected into the rats in 5 ml. doses (5 mg. SiO_2) and the rats were killed in the several experiments 3 hr. after injection. Heart, lung, liver, spleen, kidney, muscle and bone tissue was removed for analysis.

The ^{31}Si content of the several tissues are given in Table 1. No large differences are revealed between liver, spleen, lung and bone tissue. Muscular tissue (heart and gastrocnemius) have the lowest ^{31}Si content whilst the ^{31}Si content of the kidney tissue is some ten times greater than any other.

Effect of the tissue/solvent ratio on the quantity of silicon extracted by ethanol. Four rats were each injected with 8 ml. of the labelled silicate solution. They were killed and dissected after 3 hr. The liver tissue was pooled and a small sample taken for the assay of the total ^{31}Si.

One 8 g. sample and one 4 g. sample were dried and extracted with 50 ml. ethanol for 5 hr. Portions (10 ml.) were removed for assay at hourly intervals.

A part of the injected silicon can be extracted in ethanol. The extraction procedure adopted as standard involved the extraction of 4 g. tissue with 50 ml. of ethanol. The amount of silicon extracted is not increased when twice this volume of solvent is used.

Rate of extraction from and distribution of ethanol-soluble ^{31}Si in several tissues. Seven rats were each injected with 8 ml. of the solution of labelled silicate and were killed 3 hr. after. Spleen, heart, liver, kidney and lung tissues were separately collected and similar tissues were pooled. Part of each type was assayed for total labelled silicon, the rest being dried and refluxed with ethanol for 5 hr. At hourly intervals, samples of the extracts (10 ml.) were removed and assayed, the volume of the extracting liquid being kept constant by the addition of ethanol.

The extraction of the ethanol-soluble fraction of ^{31}Si from any of the tissues is almost complete under the conditions used after 2–3 hr. and the amount extracted after 1 hr. is, in most cases, some 70–80% of the total ethanol-soluble ^{31}Si (Fig. 1).

The fraction of the tissue ^{31}Si which is ethanol-soluble is highest for the heart and liver tissue (over 50%), and is very low for the kidney tissue (about 3%). Spleen and lung tissue give values of about 30% (Fig. 1).

Comparison of different organic liquids as solvents for tissue silicon. Several rats, each injected with 8 ml. of the solution of labelled silicate, were killed 3 hr. after injection and dissected. The livers were pooled and a portion was taken for direct assay. One portion of the remainder was dried and extracted with ethanol for 5 hr. Three similar portions were treated in an identical manner, but dioxan, ethyl acetate and ether were used as solvents. Samples of the extract (10 ml.) were removed at hourly intervals for direct assay.

Table 1. *Silicon content of tissues following the intraperitoneal injection of labelled silicate into rats and mice*

(Each rat received 5 mg., each mouse 1 mg. labelled SiO_2. Silicon found as μg. SiO_2.)

	Silicon in 1 g. undried tissue		Total silicon in organ	
Tissue	Rat	Mouse*	Rat	Mouse
Kidney	368±89 (4)†	138	720±(4)†	685
Liver	39±12 (4)	55	282±(4)	347
Spleen	34± 6 (4)	48	25±(4)	68
Lung	26± 5 (4)	23	52±(4)	39
Heart	21± 4 (3)	15	24±(3)	22
Muscle (gastrocnemius)	7± 1 (2)		29±(2)	
Bone (femur)				
Total	26		22	
Distal epiphyses	34		11	
Axial epiphyses	27		8	
Shaft	13		3	

* Tissues from eight mice pooled.

† Mean±S.E. and, in parenthesis, number of experiments.

Solvents other than ethanol will extract silicon from the tissues but they are less efficient. From liver tissue, in 5 hr. ether or ethyl acetate extract 10–15% of the total ^{31}Si, dioxan about 25% and ethanol up to 50%.

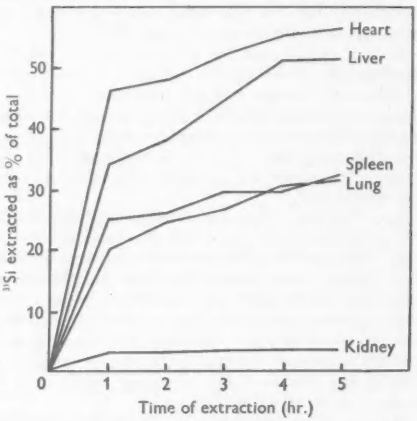


Fig. 1. Rate of extraction from and distribution of ^{31}Si in several tissues.

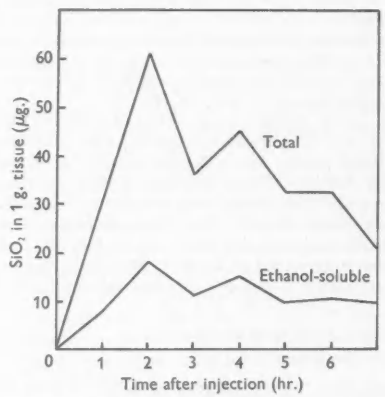


Fig. 2. Total and ethanol-soluble ^{31}Si in liver at different intervals after injection.

Comparison of the rates of increase in (a) total and (b) ethanol-soluble marked silicon in the liver after injection. Labelled silicate solution (8 ml.) was intraperitoneally injected into several rats. The rats were killed at intervals in batches of three. Pilot experiments indicated that, because of the rapid decay in the activity of the silicon, experiments could not be usefully carried out in which the silicon remained in the animal for more than 5 hr. A part of the pooled liver tissue from each batch was assayed for total marked silicon. The remainder was dried and extracted with ethanol for 3 hr. The ethanol extract was assayed.

The total ^{31}Si in the liver rapidly rises to a maximum 1–2 hr. after the injection, then slowly falls. The ethanol-soluble ^{31}Si follows a similar course, but reaches a much smaller maximum value and falls only slightly after the second hour (Fig. 2).

Formation in vitro *of ethanol-soluble silicon complexes*

In these experiments the silicate, formed by fusion of irradiated silica with sodium carbonate as previously, was neutralized to phenol red, then diluted with phosphate buffer, pH 6·5, to a concentration of 0·1 mg. SiO_2/ml. of solution.

Addition of silicate to fresh liver. Labelled silicate solution (4 ml.) was added to sliced, freshly dissected rat liver (about 10 g.). After 3 hr. the mixture was treated by the procedure for the extraction of ethanol-soluble complexes. The extraction was continued for 5 hr. A portion of the ethanol extract (10 ml.) was removed and assayed after every hour.

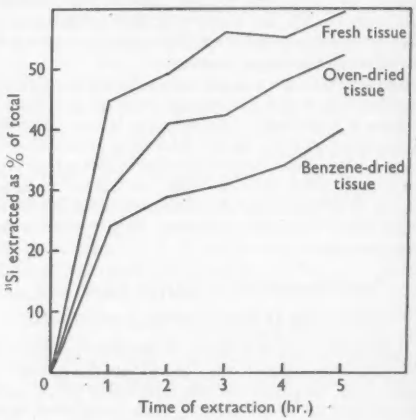


Fig. 3. Ethanol extraction of ^{31}Si added to fresh and to dried liver tissue.

The addition of silicate to excised liver tissue results in the formation of ethanol-soluble substances containing ^{31}Si. Over 50% of the added silicon can be extracted in this way (Fig. 3).

Addition of silicate to dried liver. The last experiment was repeated using liver tissue which was previously dried by azeotropic distillation.

Dried liver tissue is somewhat less effective in producing ethanol-soluble silicon complexes (Fig. 3).

Addition of silicate to ethanol-extracted liver. Rat liver was dried and given a preliminary extraction with ethanol for 6 or 24 hr. Silicate solution (4 ml.) was added to the extracted tissue which, after 3 hr., was treated by the procedure for extracting ethanol-soluble silicon complexes. At hourly intervals during 5 hr. portions of the extract (10 ml.) were removed for assay.

When the tissue is extracted with ethanol prior to the addition of silicate, the amount of silicon which is subsequently removed in ethanol is considerably diminished and the degree of the reduction varies according to the efficiency of the initial ethanol extraction. When the initial ethanol extraction is continued for 6 hr., the proportion of

the added silicon which can be subsequently extracted by ethanol is reduced to 15%; an initial 24 hr. extraction reduces the fraction to 7%.

Addition of silicate to an ethanol extract of liver. Rat liver (20 g.) was subjected to the procedure for the extraction of ethanol-soluble silicon complexes, continuing the extraction for 5 hr. Ethanol was removed from the extract by distillation under reduced pressure. Silicate solution (4 ml.) was added and, after 5 hr., the mixture was dried by azeotropic distillation. Benzene was finally removed by distillation under reduced pressure. The residue was refluxed for 5 hr. with ethanol (50 ml.), samples (10 ml.) being removed for assay at hourly intervals.

The addition of silicate to an ethanol extract of liver tissue results in the formation of ethanol-soluble complexes so that, after extraction for 5 hr., some 60% of the added silicon can be extracted (Fig. 4).

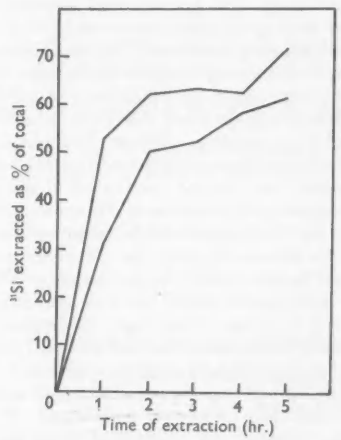


Fig. 4. Ethanol extraction of ^{31}Si added to an ethanolic extract of liver tissue.

Addition of silicate and certain other substances to ethanol-extracted liver tissue. Extraction of tissue with ethanol was shown to remove substances which acted upon added silicate. Among the substances removed by the extraction of tissue with ethanol are cholesterol, reported as forming esters with silicic acid, and phosphatides. Cholesterol and lecithin were, therefore, added to samples of ethanol-extracted tissue in an attempt to restore its property of rendering the silicate soluble in ethanol. Similar additions were made to non-protein material (cotton wool) for comparison.

Labelled silicate solution (4 ml.) was added to the ethanol-extracted rat liver (4 g.) and to cotton wool (1 g.). Cholesterol (0·4 g.), lecithin (0·4 g.), or choline chloride (0·2 g.) was added as a solution or suspension in water (8 ml.), except in the case of the blank experiments. After standing for 3 hr. the tissue was treated by the method for the extraction of ethanol-soluble silicon complexes and the ethanol extract was assayed.

Both cholesterol and lecithin added to ethanol-extracted liver tissue partially restore its ability to form ethanol-soluble silicon compounds. Cholesterol is most effective (Table 2). Replacement of the tissue by cotton wool in these experiments has little qualitative effect on the extraction except that lecithin is found to be rather more effective than cholesterol. Choline chloride is more active than either, but none of these substances is as active as the residue obtained by evaporating a benzene extract of liver tissue (Table 2).

Table 2. ^{31}Si *extracted by ethanol after the addition of* ^{31}Si *and organic substances to ethanol-extracted liver tissue and to cotton wool*

(Time allowed for reaction before drying, 3 hr. Extraction time, 3 hr. 400 μg. SiO_2 added to 4 g. extracted liver or 1 g. cotton wool.)

Substance added	Silicon extracted (μg.)	^{31}Si (% of total ^{31}Si)
(*a*) Addition to ethanol-extracted liver tissue		
Lecithin (0·4 g.)	67·5	16·9
Cholesterol (0·4 g.)	202·4	50·6
No addition	25	6·3
(*b*) Addition to cotton wool		
Lecithin (0·4 g.)	134·7	33·7
Cholesterol (0·4 g.)	69·7	17·4
Choline chloride (0·2 g.)	139	34·8
Benzene extract from 20 g. liver	180	45·0
No addition	5·8	1·5

DISCUSSION

After the introduction of labelled silicate into the peritoneal cavity of a rat, part of the silicon which reaches the tissues can be extracted with ethanol or, less effectively, with other organic solvents such as dioxan, ethyl acetate or ether. It is not extracted by benzene. The extraction is almost complete in 3 hr.

The low proportion of ethanol-soluble silicon in the kidney (about 3%) suggests that the greater part of the silicon in the kidney is in a different chemical form from that in other tissues. This silicon is presumably present as inorganic silicate, concentrated by tubular re-absorption of water from the glomerular filtrate.

The solubility in ethanol of a large part of the silicon present in other tissues supports the view that it is in some form of organic combination. The fraction of ethanol-soluble ^{31}Si in the liver reaches an approximately constant value after injection in about 2 hr. (Fig. 2), and in 1 hr. it has reached about half this value, indicating either that the organic ^{31}Si complex is rapidly synthesized or that the silicon normally present in organic combination readily interchanges with ^{31}Si.

Other experiments have shown, however, that combination of silicate with organic compounds can take place outside the body. It occurs when silicate solutions are left in contact with excised tissues, even after the tissue is dried. When tissue is extracted

with ethanol prior to the addition of silicate the quantity of 'organic silicon' produced is considerably reduced, but the ethanolic extract of the tissue itself combines with silicate (Fig. 4). These results show that the organic silicon complex is formed by a simple interaction between inorganic silicate and one or more substances present in tissues; it does not require biological activity.

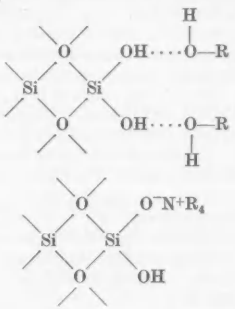
It is apparent that the 'organic silicon' fraction can be increased by the addition of substances such as lecithin, choline, or cholesterol. Moreover, the tissue is not necessary for the interaction; even higher yields are obtained when cotton wool is used as the carrier. Up to 30% of added silicate is converted into 'organic silicon' by this means (Table 2).

It is interesting that the substance which is extracted from the tissue by benzene during the azeotropic drying procedure, which was repeatedly shown to contain no silicon, will produce an ethanol-soluble silicon complex (Table 2). This indicates that, although benzene will not dissolve organic silicon complexes, the organic part of a potential complex is removed by this solvent.

Orthosilicic acid, $Si(OH)_4$, has, under normal conditions, only a transitory existence, polymerization commencing as soon as it is formed (Willstätter, Kraut & Lobinger, 1929). The silicic acid sols used in these experiments must be regarded as consisting of polymers of various molecular weights, the distribution of the particle sizes depending on the history of the solution. The structure of the silicic acid polymers is considered to be similar to that of quartz itself (Carman, 1940), and the surface consists of hydroxyl groups (Carman, 1940; Weyl & Hauser, 1951).

The rapid excretion of silicic acid following its administration by any route indicates that polymers which appear in the blood mostly pass through the wall of the kidney glomeruli and are removed. Polymers too large to be removed by this mechanism and possibly a proportion of the smaller polymers also, must be regarded as taking part in the reaction which produces ethanol-soluble complexes. The fact that such complexes can be formed *in vitro* from silicic acid and cholesterol, choline or lecithin indicates their nature.

The rapidity of the formation of the ethanol-soluble complexes, even when choline hydrochloride or cholesterol react in neutral solution, suggests that the mechanism involves no attack on the main silica framework. Combination may occur by hydrogen-bond formation between hydroxyl groups at the surface of the polymers and hydroxyl groups of the organic molecules. Complexes will also be formed by the production of polar linkages between the silicic acid, known to have a high surface potential of 300–400 mV., and ions of an opposite charge or oriented dipoles. These two types may be represented thus:



Polar linkage will be expected between silicic acid and the highly polar structures of choline and with lecithin and cholesterol. The resulting micelle will have an internal, negatively charged silicate structure surrounded by a positively charged layer of adsorbed organic ions; this layer is again surrounded by a negative, diffuse, 'gegenion' layer. The solubility of these complexes in organic solvents will depend on the proportion of (hydrophilic) hydroxyl groups of the silicic acid polymer which are blocked by the (hydrophobic) organic residues.

The formation of this type of micelle may be expected between silicic acids and all soluble biological substances which have a strongly polar group or a group which can co-ordinate with hydroxyl by hydrogen-bond formation. The multiplicity of the types of organic compounds which have been previously reported as occurring in the tissues can then be readily understood. It seems doubtful whether any reaction of silicic acid with organic molecules occurs in stoicheiometric proportions, and there is no evidence that silicon takes part in any biosynthetic process. This conception is in agreement with the observation (King & Stantial, 1933), that the total and soluble silicon values for blood are almost equal showing 'the absence of any insoluble particulate silica and of any organic compound of silicon in the blood, unless such a compound reacts directly with molybdate or is extremely susceptible to hydrolysis'. Such micelles, in the presence of strong acid, do react with molybdate.

Micelles of this type will be unable to produce the tissue damage which is caused by the action of quartz, since the outer surface of the micelle consists of organic groups and effectively isolates the silicic acid—an unusual type of detoxication. Neither a silicon-enriched diet nor large doses of injected silica sol have the deleterious effects on tissue which are produced by silica particles of the order of 1 μ. in diameter.

Silicon occurs in the tissues, then, in three forms. Smaller silicic acids exist as inorganic ions which

readily pass through the walls of the glomeruli and account for the high excretion rate when silica is ingested or injected. Large polymers of silicic acid form adsorption complexes with polar and hydroxylic organic molecules which may be soluble in organic solvents such as ethanol and dioxan. Silicon also occurs in the tissues in a form which is not excreted and is not soluble in organic solvents. The nature of these complexes is under investigation.

SUMMARY

1. The silicon in tissues has been studied by injecting into rats silicate solutions containing ^{31}Si. Muscular tissue retains the least ^{31}Si, kidney tissue the most.

2. After drying the tissues, a part of the ^{31}Si can be extracted in ethanol, dioxan or (less effectively) ethyl acetate and ether. The ethanol-soluble silicon is probably present in micelles having organic groups adsorbed on silicic acid polymers.

3. The greater part of the ^{31}Si in the kidney occurs as inorganic silicate which is not extracted by organic solvents.

4. A part of the ^{31}Si is associated with the tissue, but is not soluble in ethanol.

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Induced Loss in Cerebral Tissues of Respiratory Response to Electrical Impulses, and its Partial Restoration by Additional Substrates

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Cerebral tissues are the most susceptible of those in the animal body to lowered blood levels of glucose. Cerebral activity rapidly fails with low glucose and if severe hypoglycaemia is maintained for periods between a few minutes and an hour, recovery may not be complete when glucose is again supplied. In the present study we have sought to reproduce in separated cerebral tissues a comparable loss of excitability in absence of glucose, a loss which was not restored on adding glucose alone. These conditions having been found, restoration of excitability by other means has been sought.

Experiments of this type have become possible through the development of metabolic means of measuring response by such tissues to their electrical excitation (McIlwain, 1951*a*). Applied impulses induce various metabolic changes in cerebral tissues (McIlwain, 1952). Of these changes, the increase in respiration and lactic acid formation were chosen for the present study for the following reasons. They are large and relatively easy to determine. They give a measure of the main energy-yielding reactions of the tissue. It is understandable that failure of these reactions should lead to irreversible loss of function in the tissue.

EXPERIMENTAL

Cerebral cortex. Cerebral cortex mainly of guinea pigs was prepared and mounted for electrical stimulation as described previously, using vessels A^1 and the tissue-holding electrodes type *D* (McIlwain, 1951*a*; McIlwain & Gore, 1951). The vessels were fitted to Warburg type manometers and changes in O_2 or CO_2 were followed at 37° by the usual

manometric techniques. A vessel normally contained a slice of about 100 mg. fresh weight with 5 ml. saline. Typical experiments comprised five or six vessels containing tissue from the same animal. Creatine and inorganic phosphates were determined in certain of the slices after their rapid removal from the electrodes (McIlwain & Gore, 1951) by extraction with trichloroacetic acid and separation by Ca-ethanol precipitation (McIlwain, Buchel & Cheshire, 1951).

Salines. All salines contained 134 mM-NaCl, 5·4 mM-KCl, 1·34 mM-KH_2PO_4, 1·34 mM-$MgSO_4$ and 2·7 mM-$CaCl_2$. Glycylglycine saline contained in addition 50 mM-glycylglycine, brought to pH 7·4 by NaOH, and was saturated with O_2. Phosphate saline contained 10·4 mM-Na_2HPO_4, brought to pH 7·4 by HCl, and was saturated with O_2. Bicarbonate saline contained 26·1 mM-$NaHCO_3$ and was saturated with 95% O_2 and 5% CO_2.

Glucose and other substrates when added during an experiment were placed initially in a side arm as neutral M solutions.

an approximate doubling in respiratory rate during the period of application of the impulses.

A *and* B, *Fig.* 1. Excised blocks from the cerebral hemispheres could be kept at 0° without added substrates for several hours without loss of excitability. That is, slices later prepared from such blocks and shaken in glucose-containing oxygenated salines at 37° respired at normal rates which increased with electrical impulses. If kept at 0° for 24 hr., some fall in initial respiratory rates and in excitability occurred (McIlwain, Ayres & Forda, 1952), but this method of depleting the tissue was not examined further as the loss in excitability was only partial and the conditions involved were remote from those of hypoglycaemia *in vivo*.

C *and* D, *Fig.* 1. Conditions closer to those of the body were therefore chosen. Under normal metabolic conditions at 37° but without glucose,

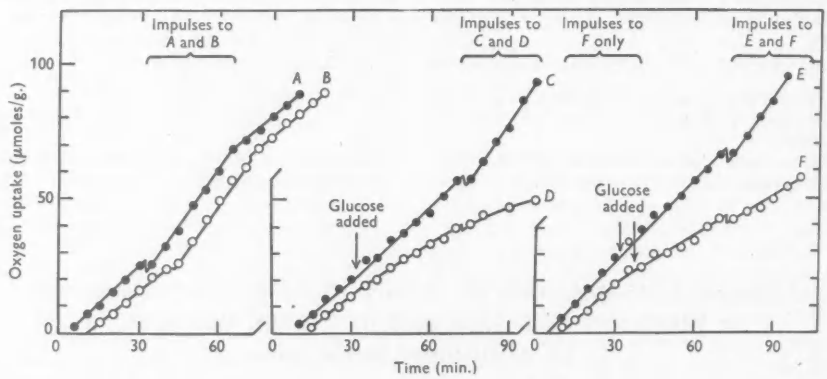


Fig. 1. Respiratory response of cerebral tissues to electrical stimulation after various treatments. Response is shown by increase in slope of the O_2-time curves. Their displacement on starting or stopping the impulses is a temperature artifact (McIlwain, 1951*a*). 5 or 10 min. have been added to the abscissae in the second of each pair of curves for clarity in the diagram. Glucose concentration, when added, 10 mM. *A*, normal tissue; and *B*, tissue kept at 0° for 2 hr., responding similarly to impulses in glucose saline. *C* and *D*, tissue in media without glucose does not show respiratory response to impulses (*D*); respiration gradually falls (*D*) unless glucose is added (*C*), when excitability also is restored. *E* and *F*, tissue initially without glucose, and impulses applied to *F*. Glucose then added to each, and impulses applied to each; *F* does not respond.

Stimulation. Except when otherwise described, stimulation was by alternating condenser pulses, 100/sec., at a peak voltage of 18 V., and of time constant 0·3–0·5 msec., from the apparatus of McIlwain (1951*a*) and later of Ayres & McIlwain (1953) which uses the same basic circuit but is supplied from a.c. mains.

RESULTS

Loss of respiratory response to electrical pulses

Fig. 1 illustrates conditions which were examined in obtaining from cerebral cortex a preparation which did not give the usual respiratory response to applied electrical impulses. The normal response is

respiration was initially at a rate slightly lower than normal, and fell with time. Applied impulses did not increase the respiratory rate. Addition of glucose after 30 min. at 37° arrested the fall. However, it also restored the ability of the tissue to respond to applied impulses.

E *and* F, *Fig.* 1. Cerebral tissues *in vivo* receive not only metabolic but also electrical influences from their environment. In examining their depletion *in vitro*, the experimental sections in absence of substrate were therefore exposed to electrical impulses of the type which would normally excite. When applied in the absence of substrate at the beginning of an experiment these led to little

Table 1. *Choice of conditions for depleting tissue*

(Guinea-pig cerebral cortex (about 100 mg.) was in tissue-holding electrodes immersed in 5 ml. glycylglycine-buffered salines without glucose, in manometric vessels. Glucose (0·05 ml. M) was present in the side arms and added at the beginning of the period indicated. Impulses were 100/sec., diphasic, peak voltage 18 V., and of time constant 0·4 msec. Respiration was obtained from manometric readings plotted as in Fig. 1.)

Exp.	Time and glucose (min. after placing at 37°)		Impulses (I, applied; 0, not applied)		Respiration (μmoles O_2/g./hr.)	
			a	*b*	*a*	*b*
1	0–15	None	I	0	46	44
	15–45	Added	0	0	48	44
	45–90	Added	I	I	62	70
2	0–30	None	I	0	46	46
	30–65	Added	0	0	46	46
	65–105	Added	I	I	42	70
3	0–35	None	0	0	38	37
	35–60	None	I	0	25	37
	60–90	Added	0	0	27	33
	90–120	Added	I	I	25	45
4	0–30	None	I	0	43	40
	30–60	Added	0	0	40	40
	60–120	Added	I	I	40	84

change or a slight fall in respiratory rate. If the impulses were then stopped and glucose added, little change occurred. However, an important difference was seen during the third period: impulses in the presence of glucose now failed to change the respiratory rate of the tissue.

Tissue so treated to impulses in absence of substrate constitutes the depleted preparation which is examined in the remainder of this study. Choice of the duration of treatment was made on the basis of experiments shown in Table 1. For depletion, 15 min. exposure to impulses was inadequate (Exp. 1); 30 min. was adequate in that later addition of glucose and application of impulses gave no respiratory response (Exp. 2). The intensity and type of impulses could also be varied, and some experiments in which this is done are described later. As however the period of 30 min., which was necessary for depletion with the type of impulses already employed, is of the order of time during which hypoglycaemia can become irreversible, the conditions of Table 1, Exp. 4, were considered satisfactory. The longer period in absence of substrate examined in Exp. 3 offered no advantage.

Glycolysis by depleted tissues

Lactic acid normally accumulates in oxygenated reaction mixtures containing cerebral tissues and glucose. This continued to take place with depleted tissues, though at a slightly reduced rate. Normal rates for cerebral cortex prepared in the same fashion as for the present experiments and at a concentration of about 20 mg./ml., were about 25 μmoles/g./hr. (McIlwain & Grinyer, 1950; McIlwain, Anguiano & Cheshire, 1951), but they fell during the course of an experiment. After incubation in the absence of substrate, values of 12–16 μmoles/g./hr. were found (Table 2) for the period of 60–90 min. after commencement of experiments. Thus temporary omission of glucose did not greatly affect the normal rates of glycolysis.

Moreover, glycolysis in the depleted tissue responded to applied impulses. A three- to five-fold increase is suggested by the values of Table 2. Exps. 1*d*, *e*, 2*d* and *e* show the formation of lactic acid to be little affected by the initial application of impulses which induced failure in respiratory response. This is shown also in Fig. 2. Here, increase in pressure, mainly due to formation of lactic acid, is seen to commence in depleted as well as normal tissue on addition of glucose, and to be accelerated by applied impulses.

The rate of lactic acid accumulation which was induced in reaction mixtures with the depleted tissues by the impulses varied with the buffer employed. It was higher with phosphate than with glycylglycine or bicarbonate (Table 2). In phosphate, the observed rate of 85 μmoles/g./hr. approached the maximum values obtained by electrical and other stimulation of cerebral cortex *in vitro*. This is of the order of 100 μmoles/g./hr. (McIlwain, Anguiano & Cheshire, 1951).

Partial restoration to depleted tissues of respiratory response to electrical impulses

One interest in the study of the depleted tissue lay in possible restoration of its full response to applied impulses. This was attempted by adding further substances to the glucose saline which by itself was adequate for response by normal tissue.

Table 2. *Lactic acid formation by treated tissue*

(Tissue and impulses were as in Table 1 except that phosphate replaced glycylglycine as buffer in Exp. 2, and bicarbonate in Exp. 3. Each vessel of each experiment was run for the three periods of 30 min. each, then promptly removed from the thermostat and samples taken for lactic acid. Each value given for lactic acid is the average of duplicate determinations; when two values are given they are from vessels run in parallel. The rate of lactic acid formation has been assumed to be uniform during each period and was calculated as follows. Taking Exp. 1 as example: *a*, 2/3 of observed value; *b*, 3·2+2 (6·4–4·7); *c*, 3·2+(14–4·7); *d*, 12·5+2 (28–14); *e*, 12·5+2 (30·5–14).)

	Conditions			Lactic acid	
Exp.	1st period (I, impulses)	2nd period (G, glucose added)	3rd period (I, impulses)	Total formed during exp. (μmoles/g.)	Calculated rate of formation during last period (μmoles/g./hr.)
1*a*	—	—	—	4·7	3·2
b	—	—	I	6·4	6·6
c	—	G	—	14	12·5
d	—	G	I	28, 28	40·5
e	I	G	I	30, 31	45·5
2*a*	—	—	—	9·8	6·6
b	—	—	I	8·4	3·8
c	—	G	—	20	16·8
d	—	G	I	50, 57	83·8
e	I	G	I	53, 55	84·8
3*a*	—	—	—	11	—
b	—	—	I	8	—
d	—	G	I	44	—
e	I	G	I	32	—

Additions included (1) substances described as liberated from nervous structures on excitation, (2) substances concerned with oxidative and not glycolytic reactions with glucose, and (3) various other materials from natural sources.

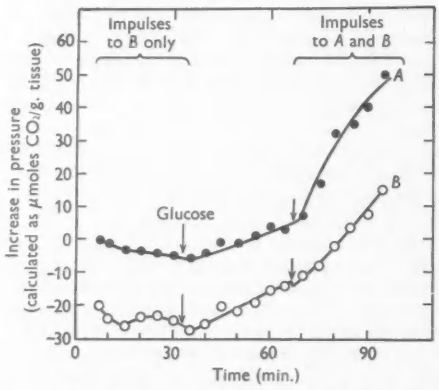


Fig. 2. Change in pressure on applying impulses to normal (*A*) and depleted (*B*) guinea-pig cortex in bicarbonate saline. Changes have been calculated as apparent μmole CO_2/g., using the K_{CO_2} of the vessels, and are largely due to the formation of lactic acid (cf. McIlwain, Anguiano & Cheshire, 1951). For clarity in the diagram, 20 μmoles have been subtracted from each ordinate of curve *B*.

An initial survey of such substances gave results which are summarized in Table 3. None of the substances of groups (1) or (3) of the preceding paragraph was effective; the first clearly active compound encountered was fumaric acid. The restoration of respiratory response brought about by fumaric acid was not complete, when examined under the conditions of depletion and testing which had been chosen on the basis of the experiments of Table 1. The conditions of depletion and of testing the tissue for respiratory response were therefore varied, and examples are given in Table 4. No effect of fumarate was found when (Exp. 1) conditions of depletion were mild, using a relatively low voltage and brief impulse. Here the tissue was relatively little depleted. The effect was seen when the impulses used were longer (Exp. 2) or of higher voltage (Exps. 3–5); the testing impulses were also varied in these experiments. No set of conditions led to a markedly greater effect of fumarate than was seen under the standard conditions adopted earlier. The results of Table 4 however showed that partial restoration of the depleted tissue by

Table 3. *Attempted recovery of metabolic response with various additional substances*

Substances (mM) *changing respiratory response by* <10%
Thiamine, 1·5; nicotinamide, 2·0 and 20; diphosphopyridine nucleotide, 0·1; adenylic acid, 10; adenosinetriphosphate, 10; choline chloride, 10; acetylcholine chloride, 0·5 and 5.
Guinea-pig and human blood serum, 10%; a 1:2 aqueous extract of guinea-pig brain, 10%.
Succinate, 10 and 40; glutamate, 10.
[*Substances* (mM) *increasing respiratory response by* >10%
Fumarate, 10 and 20; malate, 10 and 40.

Table 4. *Depletion and partial recovery with fumarate under various conditions*

(The main part of each experiment comprised measurement of respiratory rate of four pieces of cerebral tissue from the same animal during three successive experimental periods. Impulses were applied during the first period (to deplete) and during the last (to test); they were the same in each four vessels of any one period of an experiment but were altered from experiment to experiment and sometimes in the depletion period were different from those of the testing period. During the first period no substrate was present; it was tipped in a few minutes after impulses were switched off at the beginning of the second period and was either 0·01 M-glucose alone (in two of the vessels) or 0·01 M-glucose plus 0·01 M-fumarate (in the other two). Rates during the first period are not recorded, as the period was sometimes too brief to give dependable values; when obtained, values were comparable with those of Table 1.)

	1st period			2nd period		3rd period		Rate of respiration		
	Impulses					Impulses			3rd period	
Exp.	Duration (min.)	Peak potential (V.)	Time constant (msec.)	Duration (min.)	Fumarate added	Peak potential (V.)	Time constant (msec.)	2nd period (μmoles/ g./hr.)	(μmoles/ g./hr.)	Change from 2nd period (%)
1	15	14	0·1	45	0	20	0·3	52	79	+52
	15	14	0·1	45	+	20	0·3	62	99	+60
	30	14	0·1	30	0	20	0·3	49	73	+49
	30	14	0·1	30	+	20	0·3	44	58	+32
2	25	14	0·5	30	0	18	0·3	43·5	47	8
	25	14	0·5	30	+	18	0·3	54	69	28
	50	14	0·5	30	0	18	0·3	36	29	−19
	50	14	0·5	30	+	18	0·3	38	37	−2
3	25	20	0·1	55	0	20	0·1	48	48	0
	25	20	0·1	55	+	20	0·1	34	40	17
	50	20	0·1	30	0	20	0·1	45	48	7
	50	20	0·1	30	+	20	0·1	44	50	14
4	25	20	0·13	50	0	20	0·4	39	47	21
	25	20	0·13	50	+	20	0·4	55	87	57
	45	20	0·13	30	0	20	0·4	30	36	20
	45	20	0·13	30	+	20	0·4	33	38	16
5	30	18	0·4	30	0	18	0·4	33	35	6
	30	18	0·4	30	+	18	0·4	31	38	23
	30	18	0·4	30	0	18	0·4	42	44	5
	30	18	0·4	30	+	18	0·4	52	64	23

fumarate was not dependent on narrowly defined conditions of preparing and testing the tissue.

The results of Table 4 illustrate points which must be considered in expressing the ability of substances to restore excitability. Metabolic response to impulses has previously been expressed as a percentage of the rate immediately prior to their application, rather than by subtraction of one rate from the other. Under different experimental circumstances the percentage increase gives more consistent values for the effect of a given impulse. This applies to effects on tissue from different parts of the brain and from the brain of different animal species (McIlwain *et al.* 1952) and at different temperatures (unpublished). The last column of Table 4 accordingly gives such percentage changes in respiratory rate in reaction mixtures sometimes with and sometimes without fumarate. Fumarate has been considered to restore respiratory response when the percentage increase in presence of fumarate is greater than in its absence. Fumarate in some cases increased slightly the respiratory rate during the second experimental period, used as denominator in calculating the percentage. This fashion of expressing results would in such cases tend to minimize the restoring effect of fumarate. Similar considerations apply to the values of Table 5. Here, the significance of the difference between stimulation with and without added substance has been expressed as P values calculated by applying the t test. In doing this, the difference chosen has been that between the two percentage responses in a given experiment, and not that between the mean response, with and without addition, in the group of experiments with a given substance. This is because, in a given experiment, variable factors (e.g. the animal, time and temperature of preparing the tissue) operated equally on the experimental vessels with and without added substrate.

Other carboxylic acids, metabolically related to fumarate, were examined for restoration of the depleted tissue. L-Malate at 0·04 M had an effect comparable to fumarate (Table 5) but others had little or none. The respiratory response to impulses achieved by either of these compounds was about one-third of the normal response.

Inorganic and creatinephosphates in depleted tissues

The labile phosphates have already been determined in cerebral tissues prepared for metabolic work by slicing in the fashion used in the present experiments (McIlwain, Buchel & Cheshire, 1951; McIlwain, 1952; Kratzing, 1953). The creatinephosphate of the tissue from guinea pigs was then

Table 5. *Various added substances on recovery of respiratory response*

(The experimental arrangement was that of Table 4, with three successive 30 min. periods in each experiment. During the first and last impulses of 18 V. peak potential and 0·3–0·4 msec. time constant were applied. Glucose (to 0·01-M) with or without additional substrate was added at the end of the first period.)

Additional substrate (mM)	Change of respiratory rate in 3rd period (% of rate in 2nd period)		*P* (see text, p. 309)
	Without additional substrate	With additional substrate	
Fumarate, 10	6, 5, 8, 11	23, 23, 28, 25	0·01–0·001
Fumarate, 20	20, 23, 9	37, 30, 17	0·05–0·02
L-Malate, 10	8, 0, 3, 9	18, 0, 8, 8	0·3–0·2
L-Malate, 40	14, 19, 7, −4	29, 33, 21, 31	0·05–0·02
Succinate, 10	12, 4	6, 2	—
Succinate, 40	6, 16, 8, 6	0, 18, 10, 7	—
Pyruvate, 10	3, 17, 5	−5, 8, 3	—
Lactate, 10	−6, 7	−11, 10	—
Lactate, 40	7, 11	8, 23	—
Citrate, 10	20	24	—
Citrate, 20	11, 23	12, 18	—
L-Glutamate, 10	6, −2, 10	0, −12, 8	—

Table 6. *Inorganic phosphate and creatinephosphate in depleted cerebral tissues*

(Tissues, vessels, and impulses were as described in Table 1; media differed only as described below. Glucose was added to 10 and fumarate to 20 mM.)

Treatment			Tissue constituents at end of experiment	
First period of 30 min.				
Glucose	Impulses	Second period of 30 min.	Creatine-phosphate (μmoles/g.)	Inorganic phosphate (μmoles/g.)
0	0	No second period	0·31, 0·33	4·86, 5·52
0	+	No second period	0·32, 0·37	4·62, 5·60
+	0	No second period	1·52, 1·76	4·32, 3·85
+	+	No second period	0·42, 0·35	4·63, 5·05
0	0	With glucose	1·72, 1·44	3·53, 4·13
0	+	With glucose	1·44, 1·31	4·6, 4·88
0	0	With glucose and fumarate	1·26, 1·62	3·73, 4·09
0	+	With glucose and fumarate	1·29, 1·25	3·74, 3·9

shown to fall from its *in vivo* value of about 3 μmoles/g. to less than 1 μmole/g. on slicing and transferring to a physiological saline. If the slices were caused to respire in oxygen in salines without added substrate, creatinephosphate levels of about 0·3 μmole/g. were established. This is the condition of the tissue at the beginning of the present experiments (Table 6).

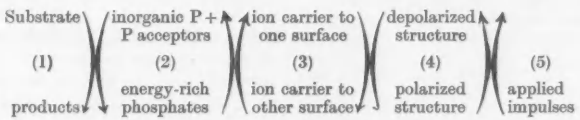
Addition of glucose-containing salines to tissue which had been without added substrates for some 10 min. after preparation, led to resynthesis of a large proportion of the creatinephosphate which it originally contained. Typical levels attained *in vitro* were of 1·5 μmoles/g. It was not however known whether this resynthesis took place in tissues which had respired for some 40 min. in absence of substrate, either with or without the application of impulses. This was the condition of the tissue in the present experiments at the end of their first period of metabolism. The results of Table 6 show that resynthesis did take place under these conditions. The levels of creatinephosphate attained did not greatly differ from those found when metabolism was throughout in glucose-containing solutions. Moreover, resynthesis to an only slightly smaller extent occurred following addition of glucose to tissues which had been depleted by impulses in its absence.

Lack of respiratory response in the depleted tissue was thus not due to lack of creatinephosphate. The addition of fumarate together with glucose, a mixture which partly restored the respiratory response, did not greatly affect the level of resynthesized creatinephosphate.

The inorganic phosphate of the slices showed under the conditions investigated (Table 6) relatively small changes, usually in a sense opposite to those of the changes in creatinephosphate.

DISCUSSION

In assessing the way in which tissues unresponsive to electrical impulses might differ from those which respond normally, it is necessary to have some picture of the normal linkage between applied impulse and metabolic response. Ideas presented previously (McIlwain, 1951*a*, *b*; McIlwain & Gore, 1951) may be summarized diagrammatically as follows:



Each of the numbered systems is of considerable complexity and the scheme is considered in more detail elsewhere (McIlwain, 1953).

In the depleted tissue, substrate is provided and labile phosphates are not seriously abnormal. The transport system (3) is presumably capable of maintaining a polarized membrane, as the glycolytic response to applied impulses is normal. Some stage of system (1) has apparently failed, and the stage is specific to respiration as distinct from glycolysis. Alternatives to this conclusion require glycolysis to be linked to functional activity independently from respiration and not through a common system such as (2) above. Failure in respiratory stages is, however, confirmed by the partial recovery following addition of fumarate or malate to glucose-containing media. The most likely role for these additions is as catalysts in the tricarboxylic acid cycle. Thus, fumarate added as the only substrate to cerebral cortical slices supports respiratory rates only a little higher than those found with no substrate. It contributes little to labile phosphates of such tissue and does not itself result in response to applied impulses (Kratzing, 1953). Alone in cerebral breis or suspensions fumarate yields pyruvate (Long, 1945) but added in catalytic quantities with pyruvate as substrate it markedly accelerates respiration (Banga, Ochoa & Peters, 1939*a*–*c*). Removal of pyruvate then increases while citrate and α-ketoglutarate accumulate (Coxon, Liebecq & Peters, 1949).

The deficiency in fumarate, induced in breis by dilution or dialysis, now appears to have been induced in the depleted tissue, and to do this both application of impulses and absence of added substrate were necessary. Both circumstances can be understood to increase the metabolism of whatever substrates are available and to increase the likelihood of mechanical loss from a tissue by its depolarization, but the manner of loss has not been investigated. Fumarate occurs in appreciable quantities in normal cerebral tissues as also does fumarase, which makes it understandable that fumarate and malate should act similarly. That citrate did not act as did fumarate may be related to a similar specificity in catalysing respiration in finely ground cerebral tissue (Banga *et al.* 1939*c*).

Requirement of additional substances for the increased respiration associated with impulses raises the question of the possible difference of such respiration from that of unstimulated tissue. Such difference seems unlikely. Loss of ability to respire more rapidly with impulses was gradual (Tables 1 and 4), and in choosing conditions for depletion the gradual loss was interrupted at an arbitrary point. That respiration can then be raised by an added catalyst could be due to increase in quantity of a catalyst already there, quite as well as by addition of one pertaining to a different metabolic route and totally absent.

SUMMARY

1. The ability of cerebral cortex slices to increase respiration with glucose as substrate, on applying electrical impulses, survived several adverse conditions. These included keeping the tissue for some hours at 0°, the prolonged passage of impulses with substrate, or metabolism in absence of substrate.

2. Adequate application of impulses in absence of substrate changed the tissue so that its respiration no longer increased when glucose was provided and impulses were applied.

3. Tissue so treated largely retained its ability to form more lactic acid from glucose in response to impulses.

4. Respiratory response could be partly restored to the changed tissue by addition of fumaric or malic acids to normally adequate metabolic mixtures. Many other additions were ineffective.

5. Inorganic phosphate and creatinephosphate have been determined in the initial and changed tissue, in the presence of various substrates, and have aided interpretation of the change induced by impulses in absence of substrate.

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The Ability of some Carboxylic Acids to maintain Phosphate Levels and support Electrical Stimulation in Cerebral Tissues

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In normal circumstances the energy requirements of mammalian brain are provided by glucose which is metabolized either completely to carbon dioxide and water or partially to lactate and pyruvate (for references, see Himwich, 1951). Glucose also enables creatinephosphate to be maintained in separated cerebral tissues (McIlwain, Buchel & Cheshire, 1951) and supports the additional respiration induced in such tissues by applied electrical impulses (McIlwain, 1951*b*). The complete oxidation of glucose in cerebral tissues can proceed through the tricarboxylic acid cycle (Coxon & Peters, 1950; Coxon, 1952; for earlier work see Quastel, 1939) and the present investigations were carried out to determine whether carboxylic acids intermediate in glucose catabolism would maintain creatinephosphate or allow response to applied impulses. Glutamic acid has already been examined from this point of view (McIlwain, 1951*a*; 1952*a*).

METHODS

The saline contained 0·128 M-NaCl, 0·0051 M-KCl, 0·00128 M-KH_2PO_4, 0·00128 M-$MgSO_4$, 0·05 M-glycylglycine (brought to pH 7·4 with NaOH) and 0·00275 M-$CaCl_2$. In some experiments, specifically mentioned in the text, this concentration of $CaCl_2$ was reduced by half and in some omitted completely; a corresponding volume of NaCl was added. In a few cases, also specially noted in the text, the glycylglycine was replaced by an equal volume of Na_2HPO_4 (brought to pH 7·4 by HCl) to give a final concentration of 0·01 M. The substrates used were either glucose or a carboxylic acid (pyruvic, succinic, α-ketoglutaric, citric or fumaric). The latter were added to the medium as solutions of the Na salt prepared by neutralizing the free acids. Glucose was added to 0·0056 M; and the carboxylic acid to 0·0056 or 0·02 M. Media were oxygenated at 37°.

Guinea-pig cerebral cortex slices were cut parallel to the outer surface and weighed as described by McIlwain (1951*b*). Slices which were to be exposed to impulses were in the type *D* tissue-holding electrodes which were used in conjunction with the type A^1 electrode vessels described by McIlwain (1951*b*). Control slices were usually freely suspended in the medium.

In most cases alternating electrical impulses were applied from a condenser as described by McIlwain (1951*b*), with a pulse frequency 100/sec., time constant about 0·1 msec., peak potential 15–20 V. Some of the slices respiring in pyruvate were activated by an applied voltage of 2·5 V. supplied by a transformer run from the 50 cyc. a.c. mains. The results of experiments using this form of excitation have been included with those obtained with condenser pulses as the responses from the two impulses were similar quantitatively.

At the end of the experiment, the slices were rapidly removed from the electrodes or vessels in the manner described by McIlwain & Gore (1951). Phosphates were determined either by a chemical method involving Ca-ethanol separation (McIlwain *et al.* 1951), or by the enzymic method described by Kratzing & Narayanaswami (1953). In a few experiments, creatine was determined as creatinine (McIlwain *et al.* 1951).

Synthetic phosphopyruvate was prepared by the method of Baer & Fischer (1949). The variation in the amounts of

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creatinephosphate accumulated by pyruvate oxidation was greater than for glucose but no explanation has been found for this difference. Since there was a possibility that phosphopyruvate was being estimated as creatinephosphate, the phosphate liberated from synthetic phosphopyruvate in the course of Ca-ethanol separation was determined. This showed that only about 5% of the phosphopyruvate was measured as creatinephosphate by the Ca-ethanol procedure.

RESULTS

Respiration

Rates of unstimulated respiration of cerebral cortex slices during the first 20–30 min. after equilibration are given in Table 1. The rate of respiration with glucose as substrate was similar to that obtained earlier (Kratzing, 1951). The concentration of glucose used in these experiments gave rates of respiration comparable with that obtainable at the higher concentration of 0·013 M used by McIlwain & Gore (1951) and supported respiration without any fall in rate for the duration of the experiment.

Respiration rates with pyruvate were identical with those for glucose and were similar to values calculated from Long's (1938) results with pigeon-brain brei. With succinate, the rate of respiration was dependent on its initial concentration; 0·02 M gave decidedly higher rates than 0·0056 M. (An increase in the rate of oxygen uptake by rat-heart slices on increasing the concentration of succinate had been reported by Webb, Saunders & Thienes, 1949.) The other substrates were investigated only at 0·02 M. Of these, citrate was the only one to support a uniform rate of respiration for the duration of the experiment. Both succinate and α-ketoglutarate showed a slight drop in respiration after the first 30 min. Although slices in fumarate had an initial rate of respiration which was similar to slices with no added substrate, the former maintained a higher rate of respiration than the latter at the end of the experiment (see Table 2).

Table 1. *Mean initial respiration rates of cerebral cortex slices with various substrates*

(Slices of guinea-pig cortex were in glycylglycine saline containing 2·75 mM-Ca^{2+} at 37°.)

Substrate	Concentration (mM)	No. of determinations	Initial O_2 consumption (μmoles/g. wet wt./hr.)
Glucose	5·6	36	55·5±1·5*
Pyruvate	5·6	11	56·4±2·3
Succinate	5·6	7	78·0±3·1
Succinate	20	28	95·6±4·6
α-Ketoglutarate	20	16	67·9±1·2
Citrate	20	16	61·1±2·8
Fumarate	20	12	46·8±2·1
None	—	10	42·7±3·1

* Standard error of the mean.

Effect of electrical impulses. Respiration rates for cerebral slices with different substrates during the passage of electrical impulses are shown in Table 2. Since placing cerebral cortex slices in tissue-holding electrodes caused only minor changes in the respiratory rate of slices respiring with glucose (McIlwain, 1951*b*; Kratzing, 1951), all values for control

Table 2. *Oxygen consumption on exposure to electric current*

(Guinea-pig cerebral cortex slices were respiring in glycylglycine saline at 37°. Substrate concentration for glucose and pyruvate was 5·6 mM; other substrates were at 20 mM. The initial period was for 20–30 min. after equilibration and was followed by a period of 20–30 min. in which the 'exposed' tissue received impulses from a condenser as described in text. 'Control' tissue received no electric impulses.)

Substrate	Tissue	No. of determinations	Mean O_2 consumption (μmoles/g. wet wt./hr.) Initial period	Second period	Mean change and S.E.M (%)	Statistical significance of mean change in exposed tissue to mean change in control tissue
Glucose	Control	14	55·4	57·4	4·3±2·4	S ($P<0·001$)
	Exposed	14	53·8	94·1	74·6±8·1	
Pyruvate	Control	5	56·2	56·2	0·0±0·0	S ($P<0·001$)
	Exposed	5	56·8	92·8	76·7±6·8	
Succinate	Control	8	86·6	81·0	−6·1±2·3	NS
	Exposed	8	88·8	78·9	−10·2±4·2	
α-Ketoglutarate	Control	8	66·1	62·4	−5·5±1·8	NS
	Exposed	8	69·6	65·8	−9·2±3·2	
Citrate	Control	8	55·9	55·9	0·0±5·0	NS
	Exposed	8	66·3	61·3	−6·4±4·7	
Fumarate	Control	6	45·0	38·5	−14·2±3·7	NS
	Exposed	6	48·7	37·7	−16·9±3·5	
None	Control	4	37·0	25·7	−27·9±10·0	S ($P<0·05$)
	Exposed	4	43·5	26·5	−39·4±7·2	

S, significant; NS, not significant.

slices, whether held in electrodes or free, have been combined. The statistical significance of observed changes in exposed slices compared with those for control slices has been estimated by the *t* test.

Respiration of cerebral slices metabolizing glucose or pyruvate showed a significant increase ($P < 0.001$) over the control slices during exposure to impulses. No other substrate used was able to support the respiratory response to electrical impulses. Indeed it appeared that electrical impulses caused a slight depression of respiration. This was marked in the experiments with no added substrate.

Effect of Ca^{2+}. Since citrate combines with Ca^{2+}, experiments were undertaken to find the effect of low calcium concentrations on the rate of respiration of cerebral cortex slices during electrical stimulation. Dickens & Greville (1935) and Krebs & Eggleston (1938) have shown the inhibitory effect of Ca^{2+} on respiration, and recently Gore & McIlwain (1952) have shown that reduced Ca^{2+} concentrations in the medium caused an increased rate of respiration in cerebral cortex slices.

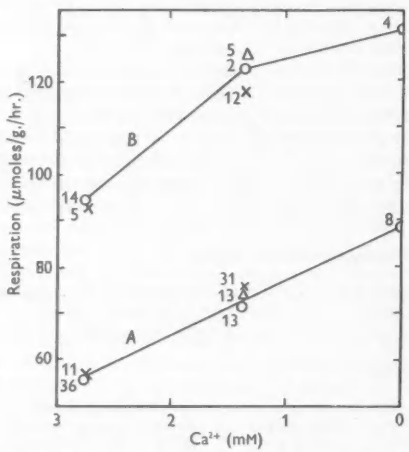

Fig. 1. Respiration of cerebral tissues and Ca^{2+} content of media. *A*, mean values in experiments with sections which did not receive electrical impulses; *B*, mean values in experiments with sections which received electrical impulses as described in text. ○, 5·6 mM-glucose with glycylglycine buffer; ×, 5·6 mM-pyruvate with glycylglycine buffer; △, 5·6 mM-pyruvate with phosphate buffer. The numbers adjacent to points denote the number of experiments in each group.

The respiratory rates of stimulated and unstimulated cerebral slices have been measured in glucose- or pyruvate-containing media in which the Ca^{2+} concentrations have been varied. Fig. 1 shows that the rate of respiration of unstimulated slices, with glucose as substrate, increased with lowered Ca^{2+} concentrations. The same results were obtained with pyruvate as substrate. On electrical stimulation the greatest response to the impulses was obtained with less Ca^{2+} than the 0·00275 M normally used.

Determination of phosphates

Inorganic. The mean values of the inorganic phosphate content of cerebral cortex slices, obtained by pooling the results of several experiments are shown in Table 3. The values obtained with glucose are within the range reported by McIlwain *et al.* (1951). Cerebral cortex slices respiring in pyruvate had a significantly greater inorganic phosphate content than slices in glucose. The highest inorganic phosphate concentrations were obtained with slices in succinate media.

Table 3. *Inorganic phosphate content of slices under various conditions*

(Pooled results from several experiments. The values for phosphates are adjusted for the inorganic phosphate content of adhering saline as described by McIlwain *et al.* (1951).)

Substrate (mM)	Ca^{2+} in medium (mM)	No. of determinations	Mean inorganic phosphate content, ± standard error (μmoles/g. wet wt.)
Glucose, 5·6	1·38	9	3·43 ± 0·15
Glucose, 5·6	2·75	4	3·28 ± 0·27
Pyruvate, 5·6	1·38	15	4·22 ± 0·22
Pyruvate, 5·6	2·75	6	4·14 ± 0·38
Succinate, 5·6	2·75	7	6·40 ± 0·27
Succinate, 20	2·75	10	8·02 ± 0·25

Energy-rich phosphates. The enzymic method of phosphate analysis described by Kratzing & Narayanaswami (1953) has been used to measure the ability of some carboxylic acids to maintain the energy-rich phosphate content of cerebral cortex slices as well as to study the effect of electrical impulses on the levels of this phosphate. In these studies the energy-rich phosphates determined were creatinephosphate (CP), adenosinetriphosphate (ATP), and adenosinediphosphate (ADP) since together these compounds account for most of the energy-rich phosphates of tissue. Results obtained by this method of analysis for the phosphate content of cerebral cortex slices respiring in glucose, citrate, succinate or fumarate media are given in Table 4. This method is not suitable for the estimation of phosphates under all circumstances (see Slater, 1953) and could not be used for experiments with pyruvate. It was also found unsatisfactory for the experiments with α-ketoglutarate.

Glucose maintained the greatest amount of energy-rich phosphate in the slices (3·01 ± 0·18 μmoles/g. wet weight tissue). Citrate was the most effective of

Table 4. *Effect of substrates and electrical impulses on phosphates of cerebral cortex slices*

(Guinea-pig cortex slices were respiring under conditions described in Table 2. The phosphates were determined by an enzymic method. The electrical impulses were condenser discharge, as described in text. The 'exposed' tissue received the electrical impulses.)

Substrate	Tissue	No. of determinations	Total energy-rich phosphate (±standard error) (μmoles/g. wet wt.)	Creatine-phosphate (μmoles/g. wet wt.)	Adenosine-triphosphate (μmoles/g. wet wt.)	Adenosine-diphosphate (μmoles/g. wet wt.)
Glucose	Control	6	3·01±0·18	1·11	0·89	0·02
	Exposed	6	1·96±0·13	0·63	0·63	0·11
Citrate	Control	4	1·68±0·15	0·87	0·32	0·17
	Exposed	4	1·02±0·11	0·69	0·06	0·08
Succinate	Control	3	0·87±0·02	0·45	0·07	0·25
	Exposed	3	0·72±0·05	0·42	0·05	0·21
Fumarate	Control	3	0·76±0·13	0·44	0·12	0·11
	Exposed	3	0·66±0·08	0·38	0·09	0·09
No substrate	Control	2	0·66	0·37	0·13	0·03
	Exposed	2	0·66	0·41	0·11	0·02

the carboxylic acids tested in maintaining the amount of energy-rich phosphate. Despite the high oxygen consumption of slices in a succinate medium, the energy-rich phosphate content of the slices was found to be 0·87 μmole/g. wet weight tissue, a value only slightly higher than the 0·66 μmole/g. wet weight tissue found in slices with no added substrate. (Cross, Taggart, Covo & Green (1949) have found that succinate oxidation supported a much lower esterification of phosphate than other substrates in washed particles of kidney and liver.) Slices respiring in fumarate showed little difference in their content of energy-rich phosphate from those with no added substrates.

Change in energy-rich phosphates. The total energy-rich phosphates of cortex slices respiring in glucose media showed a significant decrease ($P < 0·01$) from the control value after exposure to electrical impulses. The same result was obtained with slices respiring in citrate and, to a lesser extent, in succinate media. With fumarate and no added substrate, electrical impulses caused no change in the energy-rich phosphate content of the slices.

The CP content of slices respiring in glucose was 1·11 μmoles/g. wet weight tissue and electrical stimulation reduced this value to 0·63 μmole/g. tissue. These values are in good agreement with those obtained by McIlwain & Gore (1951). Citrate supported a CP content of 0·87 μmole/g. tissue. This value was reduced to 0·69 μmole/g. tissue by the passage of electrical impulses. The CP levels with succinate and fumarate were no higher than with no added substrate and were little affected by the electrical impulses.

Glucose produced a higher content of ATP than any of the other substrates, although this amount was only about half of the ATP content of rapidly frozen cerebral cortex (Kratzing & Narayanaswami, 1953). As with cortex *in vivo*, slices respiring in a glucose medium contained no ADP. Electrical stimulation caused lowered ATP content of the slice from 0·89 to 0·63 μmole/g. tissue and this was accompanied by the production of 0·11 μmole/g. tissue ADP.

Less ATP was maintained in the presence of the other substrates. The content with citrate was 0·32 μmole/g. tissue and this value was reduced by the passage of electrical impulses. With fumarate and also without added substrate, a content of approx. 0·1 μmole/g. tissue was maintained and this low value was reduced by half in slices respiring in succinate. The passage of electrical impulses produced no change in the content of ATP in these cases. All substrates except glucose caused the appearance of definite amounts of ADP. The ADP content of slices with succinate was 0·25 μmole/g. tissue and nearly double that found with citrate and fumarate. Electrical impulses caused no changes in the ADP content in these cases.

With the enzymic method of phosphate determination it was not possible to measure the energy-rich phosphates and the phosphorylated sugars in an extract prepared from 0·1 to 0·3 g. cerebral tissue. A value was obtained for the total of all the phosphorylated sugars, including triosephosphates, as well as for non-phosphorylated substances which interfere with the determination. Kratzing & Narayanaswami (1953) deal more fully with this determination called the 'blank', and with the probable range of phosphates measured. Glucose gave the greatest 'blank' value of 0·28 μmole/g. tissue which was nearly three times that for citrate, succinate and fumarate. With no added substrate the 'blank' was zero. The passage of electrical impulses did not alter the values of the 'blank'.

Some values for CP with pyruvate as substrate were obtained by a different method and are not reported in detail. They suggested that pyruvate

maintained a level of CP similar to that with glucose, and that this level fell on passage of electrical impulses.

Table 5. *Effect of succinate concentration on tissue creatinephosphate*

Substrate (M)	Creatinephosphate determined as Phosphorus (μmoles/g. wet wt.)	Creatinine (μmoles/g. wet wt.)
0·0056	1·9, 1·6, 1·0 1·4, 1·5	—
0·02	0·3, 0·3 0·3, 0·3	0·3, 0·3 0·3, 0·2

During succinate oxidation, substrate concentration appeared important for the maintenance of the CP level as shown in Table 5. The higher concentration of succinate gave a markedly lower level of CP. Furchgott & Shorr (1948) had previously found that the amount of CP in slices of dog cardiac muscle or intestinal smooth muscle was lower when succinate was added to the medium than in its absence.

DISCUSSION

Respiratory rates observed in the absence of applied potentials in the present studies are comparable with those of previous reports (Elliott, Greig & Benoy, 1937; Jowett & Quastel, 1937; Krebs & Johnson, 1937; Lipsett & Crescitelli, 1950; Quastel & Wheatley, 1931, 1932).

The new findings with respect to respiratory rates with applied impulses, and the levels of phosphate derivatives, enable suggestions to be made on how the impulses affect metabolism. Because slices respiring in pyruvate exhibit respiratory response to impulses, the glycolytic breakdown of glucose is not essential to response. Other carboxylic acids which may not allow the full functioning of the tricarboxylic acid cycle, supported neither high levels of energy-rich phosphates nor respiratory response to impulses. With citrate, where an intermediate level of energy-rich phosphate was maintained, electrical impulses lowered these without altering the respiration rate of the slice. This would indicate that the change in phosphates on stimulation is a more fundamental change than the alteration in the rate of respiration. Many previous observations have connected the labile phosphates with respiratory response (McIlwain, 1952*b*).

From the results of Brody & Bain (1952) and Abood & Gerard (1952) it is apparent that pyruvate, succinate, citrate, fumarate and glutamate are all capable of being oxidized by particulate preparations from mammalian brain and this oxidation results in an esterification of inorganic phosphate which is similar quantitatively for all substrates except citrate and succinate. Thus the low values for labile phosphate now found in slices with fumarate as substrate must be due to factors not operating in mitochondria, possibly concerning transportation to site of reaction. Rate of production of available energy-rich phosphate after other such energy requirements have been satisfied appears to determine whether a substrate can support the metabolic response to electrical impulses.

In intact cerebral cortex cells, as distinct from mitochondrial preparations, there is a limited ability to oxidize malate or fumarate (Quastel & Wheatley, 1932; Elliott *et al.* 1937). This may be due to organization of the enzymes responsible for this oxidation in the case of cells, so that in the intact cell the tricarboxylic acid cycle functions slowly unless pyruvate is present to combine with oxaloacetate and drive the malate-fumarate equilibrium forward. In other words, the tricarboxylic acid cycle may proceed at its maximal speed only when pyruvate is present. This in turn would govern the rate of production of available energy-rich phosphate and thus determine the capacity to respond to electrical impulses.

SUMMARY

1. With pyruvate (as well as with glucose) as substrate, respiration of cerebral cortex slices increased on passage of electrical impulses.

2. Respiration of slices in glucose or pyruvate was dependent on the concentration of calcium ion in the media. The two substrates gave similar respiratory rates with two concentrations of calcium ion. Electrical stimulation produced rates of respiration which were also dependent on the calcium ion concentrations of the medium.

3. Respiration of slices metabolizing 0·02M-succinate, α-ketoglutarate, citrate or fumarate was not increased by the application of electrical impulses.

4. There was a significant fall in the energy-rich phosphate content of slices respiring in glucose, citrate or succinate, during the passage of electrical impulses.

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The Enzymic Determination of Energy-rich Phosphates in Brain

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Existing methods of measuring various phosphate esters in animal tissues are open to improvement in two ways. They are not sufficiently specific for certain constituents and they require relatively large amounts of tissue to carry out a complete analysis. In order to follow phosphate metabolism in preparations of cerebral tissues such as the slices used metabolically, it has been necessary to adapt methods of Kerr (1935) and Stone (1943) to deal with minimal quantities of material (see McIlwain, Buchel & Cheshire, 1951). Moreover, when such methods are used, even with the refinements which have been introduced to adapt them for minimal quantities of material, one can obtain values only for inorganic phosphate, creatinephosphate and total easily hydrolysable phosphate. Thus it is not possible to follow changes in adenosinetriphosphate or adenosinediphosphate specifically. Recent findings of Kornberg (1950) indicate that changes in inorganic pyrophosphate during metabolism are more extensive than previously realized. Changes in easily hydrolysable phosphate, therefore, may not necessarily be a true measure even of total nucleotide phosphate.

The introduction of spectrophotometric methods of phosphate analysis, based on the enzymic formation of dihydroxyacetonephosphate and its reduction of reduced diphosphopyridine nucleotide (Racker, 1947; Slater, 1951), offered a means of following the metabolism of phosphates in small pieces of tissue with the advantage of a high degree of specificity. Since we were mainly interested in the changes in individual energy-rich phosphates (adenosinetriphosphate, adenosinediphosphate and creatinephosphate) under various metabolic conditions, we have adapted Slater's (1953) methods of phosphate determination to our particular needs for cerebral tissues and have compared analyses by this method with that of Stone (1943). Previous publications from these laboratories have reported on the ability of different substrates and metabolic conditions to support the resynthesis of creatinephosphate and other labile phosphates in respiring slices of mammalian cerebral cortex (McIlwain *et al.* 1951; McIlwain & Gore, 1951; McIlwain, 1952*a*). It was of interest to know whether similar changes took place in the adenosinepolyphosphates of the slices, and whether there was an interrelation between changes in creatinephosphate and changes in the adenosinepolyphosphates. As the phosphocreatine and adenosinepolyphosphate content of

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such slices maintained under various metabolic conditions had been determined by the calcium-ethanol separation method before the enzymic method was adapted, results with this method also are included here.

METHODS

Abbreviations. ATP, adenosinetriphosphate; ADP, adenosinediphosphate; CP, creatinephosphate; HMP, hexosemonophosphate; ~P, energy-rich phosphate; DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide.

Principles of analysis. Slater's (1953) procedure *B*(*b*) measures the total ~P content of an extract. The energy-rich phosphate compounds which react are ATP, ADP, CP and phosphopyruvate as well as phosphoglycerate (potentially an energy-rich compound). Since the phosphopyruvate and phosphoglycerate content of cerebral tissues is usually low (LePage, 1949; Stone, 1943), it can be assumed that ~P = 2ATP + ADP + CP. Slater's procedure *C* measured ATP, so that from the two measurements it is possible to determine ADP + CP. We wished to measure ADP and CP separately. This was done by modifying Slater's procedure *C* by the addition of myokinase, so that the total HMP formed equalled 2ATP + ADP, according to the following reactions: (1) 2ATP + 2 glucose → 2ADP + 2HMP; (2) 2ADP → ATP + adenylic acid.

The possible incompleteness of reaction (2), due to inhibition by adenylic acid (Colowick & Kalckar, 1943) was overcome by the addition of excess enzyme.

In order to correct for phosphorylated sugars and other possible interfering substances (e.g. pyruvate and oxaloacetate) in extracts, these substances were determined in a separate sample of extract ('blank') and the correction applied to the ATP and ATP + ADP determinations. In experiments with a pure solution of phosphoglycerate, it was found that phosphoglycerate was quantitatively estimated by the 'blank' procedure. In the case of ~P determinations it was realized that hexosediphosphate, triosephosphate, pyruvate, and oxaloacetate would be measured as well as the energy-rich phosphate compounds, but all these compounds would be determined in the 'blank', together with hexosemonophosphates. As the extracts contained little phosphorylated sugar, we have assumed all reactive material to be monophosphorylated sugar and have applied no correction to the ~P determinations. The earlier work of LePage (1946) suggested that the total monophosphorylated sugar content of brain was much greater than the sum of the hexosediphosphate and triosephosphate.

Salines and conditions of metabolism. Glycylglycine saline was used for all the metabolic experiments. The composition was the same as that described by McIlwain *et al.* (1951). Glucose was used at 0·012 M and glutamate at 0·02 M. Usually 80–120 mg. slices and 3 ml. of medium were contained in each vessel. The gas phase was O_2 in all the manometric experiments where aerobic conditions were used. For anaerobic experiments the gas phase was 95% N_2 and 5% CO_2. When 2:4-dinitrophenol was used in the experiments, the appropriate amount was added to the medium before slices were exposed to it. For measurement of phosphates after cutting, the slices were kept in the medium at room temperature until they were dropped into the deproteinizing solution. This was done within 30 min. of the death of the animal.

Preparation of extracts

Extracts from slices of guinea-pig cerebral cortex. Slices from the outer grey matter of guinea pig cerebral hemispheres were cut and weighed as described by McIlwain *et al.* (1951). The following procedure was adopted for preparation of extracts whether or not the slices had been used for manometric studies. Two slices of cerebral cortex (total 0·2–0·3 g.) with any adhering saline were dropped into 1 ml. ice-cold 19% (w/v) $HClO_4$ in a tared Potter-Elvehjem homogenizer, promptly disintegrated and reweighed. After standing for a few minutes in ice, the contents of the homogenizer tube were centrifuged at high speed in a refrigerated centrifuge and the largest possible quantity of supernatant removed, measured and neutralized to approx. pH 7·6 with a measured quantity of 5 N-KOH, using phenol red as an external indicator. The addition of a small volume of glycylglycine buffer, pH 7·6, appreciably helped the final adjustment of pH. The neutralized extracts were stored at −20° until analysed. All analyses were based on the initial fresh weight of the tissue. For analyses by the Ca-ethanol method, two slices of cortex were similarly added to tared homogenizer tubes containing 3·0 ml. ice-cold 10% (w/v) trichloroacetic acid, reweighed and the contents disintegrated and centrifuged in the cold after a few minutes standing in ice. The maximum practicable volume of the supernatant was removed, measured and subjected to the Ca-ethanol procedure.

Extracts from rapidly frozen brain. Young guinea pigs, weighing 80–100 g., were dropped head first into about 500 ml. liquid air in a wide-mouthed thermos flask. When the vigorous boiling which this produced had ceased, the animal was judged to have reached the temperature of liquid air. It was removed from the flask, the head scraped to remove the fur and the skull opened by a medial split with a ¾ in. chisel cooled in liquid air. The skull was prised open to expose the cortex from which pieces were chipped. During this operation the tissue was immersed in liquid air every few seconds. The chips of brain (mainly cortex) were either added to ice-cold 19% (w/v) $HClO_4$ and disintegrated as already described or ground to a fine powder under liquid N_2 in a Wedgewood mortar and approx. 200–300 mg. powder added to each homogenizer tube. In one experiment, the animals were guillotined; the heads were immersed in liquid air within 1 sec. of decapitation. The frozen brain was then removed as before.

Enzyme preparations

Myokinase was prepared by the method of Colowick & Kalckar (1943). The material precipitated by 80% saturation with $(NH_4)_2SO_4$ was redissolved and stored in the frozen state. It was diluted 1:10 before use. Other enzyme preparations and DPNH were as described by Slater (1953). The activity of the enzymes was of the order obtained by Slater. We are grateful to Dr B. A. Askonas for the gift of creatinephosphokinase used in this work.

Method of preliminary incubation for determination of ATP and ADP

The method of incubation for ATP was similar to that described by Slater (1953) except that the 0·15 ml. 0·25 M-NaF was replaced by 0·15 ml. 0·043 M-ammonium molybdate. In experiments undertaken to compare fluoride and

molybdate as inhibitors of phosphatases in this incubation, it was found that molybdate was as efficient as fluoride for this purpose. With the hexokinase preparation used, 2 min. at room temperature was found to be an adequate incubation time after which 0·32 ml. 38% (w/v) $HClO_4$ was added to deproteinize the solution. After centrifuging, the supernatant was neutralized with 5N-KOH. $HClO_4$ was considered preferable to trichloroacetic acid because, after deproteinization, the former could be nearly completely removed as the K salt. It was important to keep these neutralized extracts ice-cold while samples were being taken for spectrophotometric measurements.

ADP was converted to HMP under the same conditions as those used for ATP. In addition to hexokinase, the incubation mixture contained 0·05 ml. of dilute myokinase, prepared from the stock solution by diluting 0·1 ml. to 1·0 ml. Incubation for 4 min. was found necessary for complete reaction.

Spectrophotometric measurements

Changes in the optical density, caused by reaction of DPNH with the dihydroxyacetonephosphate eventually formed, were measured in a Beckman model DU quartz spectrophotometer as described by Slater (1953). For the determination of ~P in the extract, 0·1 ml. creatinephosphokinase (0·2 mg./ml.) was added to the spectrophotometer cells in addition to the reagents described in Slater's procedure *B*(*b*).

Chemical determination of tissue phosphates

Inorganic phosphate, CP, and adenosinepolyphosphate were determined in both slices and frozen tissue by Ca-ethanol separation followed by colorimetric determination of the inorganic phosphate according to the method of Stone (1943). In the case of extracts from slices, hydrolysis of the Ca-insoluble fraction in N-HCl was carried out on separate samples for 10 min. and 30 min. The former gave the value for adenosinepolyphosphate and the latter an indication of other hydrolysable substances present in the fraction. All volumes recommended by Stone were reduced to one-eighth so as to adapt the method to the smaller quantity of tissue available. For comparison of the present method with that of Stone, a pooled $HClO_4$ extract was prepared from powdered frozen brain and neutralized with KOH as already described. Before separation was begun, trichloroacetic acid was added to the neutralized extract in the concentration used by Stone.

RESULTS

Analysis of various phosphate compounds

To test the efficiency of the myokinase modification for separate measurement of the adenosinepolyphosphates, it was applied to a laboratory preparation of ATP of high purity and also to a preparation expected to consist largely of ADP (Table 1).

When both hexokinase and myokinase were used for the preliminary incubation, twice as much HMP was formed as on incubation with hexokinase alone. The value for 2ATP+ADP agreed closely with the ~P value obtained by Slater's procedure *B*(*b*). It has been reported by Slater (1953) that most laboratory preparations of ATP contain insignificant amounts of ADP, and the results of this analysis therefore indicate that the second phosphate group of ATP is being estimated quantitatively by this method.

The ADP preparation had been obtained from ATP by Bielschowsky's (1950) method. Again the total adenosinepolyphosphate value obtained by Slater's procedure *B*(*b*) and by the myokinase procedure were in close agreement and both led to ADP values also in close agreement. The ATP content was higher than expected, an observation also reported by Slater (1953) for ADP prepared by this method.

Recovery experiments were carried out using a synthetic sample of sodium creatinephosphate. The sample was free from inorganic phosphate and contained 8·0% total phosphorus (calc. for the hexahydrate, 8·5%). Determination of CP after calcium ethanol separation gave 7·95% CP phosphorus. The CP was determined as the increase in the ~P content of a solution of either ATP or ADP on addition of CP and creatinephosphokinase. The

Table 1. *Comparison of methods of analysis*

(Analytical methods are described in the text. The ATP was a laboratory preparation received from Dr E. C. Slater. The ADP was prepared from commercial ATP by Bielschowsky's (1950) method. The CP, a laboratory preparation, was dissolved to give two solutions (*a*) and (*b*).)

		Values by enzymic method (μmoles/ml.)					
						ADP	
Preparation	Values obtained by chemical determination of acid-labile P (μmoles/ml.)	~P	CP	ATP	2 ATP +ADP	2 ATP+ADP −2 ATP	~P−2 ATP
ATP	—	46·0	—	22·6	47·1	1·9	0·8
ATP	—	44·8	—	22·4	45·2	0·4	0·0
ADP	7·33	6·16	—	1·97	6·20	2·26	2·22
ADP	—	6·26	—	1·97	6·26	2·32	2·32
CP (*a*)	4·25	—	4·06	—	—	—	—
CP (*a*)	4·25	—	4·10	—	—	—	—
CP (*b*)	17·37	—	15·50	—	—	—	—
CP (*b*)	17·37	—	16·10	—	—	—	—

Table 2. *Comparison of methods of analysis for determining the phosphate content of frozen brain*

(Methods of determination are described in the text. Pooled extracts from the same young guinea pigs (80–100 g.) were used for comparison of the two methods.)

Method of killing	Enzymic method (μmoles/g. fresh tissue)					Ca-ethanol separation (μmoles/g. fresh tissue)				
	~P	CP	ATP	ADP	'Blank'	Sum of CP + adenosine-poly-phosphate	CP	Adenosine-polyphosphate	Inorganic P	Total P
Whole animal dropped into liquid air (*A*)	8·41, 8·56, 8·66, 8·58	2·14	3·28, 3·18	0·0, 0·0	0·26	10·06	2·66, 2·74	7·35, 7·19	3·58, 3·67	26·7
Guillotined; head dropped into liquid air (*B*)	8·30, 8·16, 7·82, 8·09	1·96	3·10, 3·04	0·0, 0·0	0·33	10·36	1·75, 1·85	8·56, —	5·04, 4·91	29·4

recoveries obtained under these conditions agreed well with the amounts expected from the phosphorus content of the material. With the enzymes at our disposal Slater's procedure *C*, with the inclusion of creatinephosphokinase in the incubation mixture, proved unsatisfactory; only 50–60% of the CP could be accounted for as increased HMP even after prolonged incubation. The direct determination of CP would have been preferred to that of calculation by difference, but in view of the poor recoveries obtained, the latter method was used in all further experiments.

The phosphate content of frozen cerebral tissue

In order to check in biological material the results obtained by these methods of analysing phosphates, extracts of rapidly frozen brain (see Methods) were prepared and analysed both enzymically and by the calcium-ethanol separation of Stone (1943). Results are given in Table 2.

Values for the CP content obtained by the two methods are of the same order for both extracts. The value for CP as determined by the chemical method on extract *A* are comparable with those obtained by McIlwain *et al.* (1951) for guinea-pig brain cortex. However, only 80% of this value was measured enzymically. In extract *B*, the enzymic CP determination gave a slightly higher value than the chemical method. There was no detectable amount of ADP in either extract. The sum of the CP and adenosinepolyphosphate obtained by the chemical method is 1·5–2·2 μmoles/g. higher than the ~P value determined enzymically. This difference may possibly be due to the presence of substances like inorganic pyrophosphate which are separated and determined in the adenosinepolyphosphate fraction but are not measured by the more specific enzymic method.

The 'blank' value reported in Table 2 is a measure of the total hexosemono- and di-phosphates, triosephosphates and phosphoglyceric acid as well as of pyruvate and oxaloacetate. By independent analysis using lactic dehydrogenase, only barely detectable traces of pyruvate were found. The constituents of this fraction were not further differentiated. These values are lower than those previously reported by Stone (1943), LePage (1946) and Dawson & Richter (1950).

The values for brain constituents obtained by Kerr (1935) and Dawson & Richter (1950) indicate that freezing the whole animal causes less stimulatory activity in the animal than other methods of killing, such as decapitation. The present findings (Table 2) are in agreement with these observations.

Phosphates of cerebral cortex slices

The results of several experiments in which the phosphates of cerebral cortex were measured are given in Tables 3 and 4. The resynthesis of creatinephosphate reported to occur after respiration in glucose-containing saline (McIlwain *et al.* 1951) has been confirmed by both methods of analysis. While the level of CP increased two- to three-fold and that of inorganic phosphate decreased significantly, there was a far smaller increase in the ATP content and a fall in the 'blank' value. No significant amounts of ADP could be found in slices either before or after respiration in glucose saline. It was of interest, however, to find that in the presence of 0·05 mM-2:4-dinitrophenol, simultaneously with a failure to resynthesize CP and a fall in ATP content, significant amounts of ADP were formed. The same observation was made with slices respiring in glutamate saline, but phosphate values obtained on extracts from them using the enzymic method tended to vary widely and are not reported here. Due to the presence of glutamate or some metabolic product formed from glutamate in these extracts, the enzymic method of determining phosphates was not entirely satisfactory. The oxidation of DPNH normally continues to take place at a slow rate throughout the period of spectrophotometric

Table 3. *Phosphate content of cerebral cortex slices by enzymic method*

(Slices were cut in glycylglycine saline with 0·012 M-glucose and incubation, when carried out, was in the same medium for 60 min. Mean values are given ± standard error; number of determinations are given in parentheses.)

Respiration (μmoles/g./hr.)	2:4-Dinitrophenol in medium (mM)	Phosphates (μmoles/g.) ~P	CP	ATP	ADP	'Blank'
(Slices after cutting, not incubated)	0	2·15±0·07 (3)	0·68±0·13 (3)	0·73±0·17 (3)	0·00±0·02	0·35±0·02 (3)
55·5±1·5	0	3·12±0·14 (12)	1·12±0·04 (6)	1·01±0·09 (5)	0·01±0·04 (4)	0·23±0·02 (5)
107·5±6·5	0·05	1·52 (2)	0·18 (2)	0·57 (2)	0·19 (2)	0·16 (2)

Table 4. *Phosphate content of cerebral cortex slices by* Ca*-ethanol separation*

(Respiration was for 60 min. in glycylglycine medium with 0·012 M-glucose or 0·02 M-glutamate and anaerobic glycolysis for 60 min. in bicarbonate saline with 0·012 M-glucose. Methods of determination and experimental details are given in text. Inorganic phosphate values (as μmoles/g. wet weight of tissue) and the standard error are given.)

Experimental conditions: Substrate	2:4-Dinitrophenol (mM)	Respiration or glycolysis (μmoles/g./hr.)	Phosphates (μmoles/g.): Inorganic P	CP	Adenosinepolyphosphate (10 min. hydrolysis)	Phosphate liberated on 30 min. hydrolysis	Adenosinepolyphosphate + CP
Glucose	0	(After cutting, not incubated)	7·15±0·54 (4)	0·45±0·12 (3)	1·79±0·09 (4)	2·59±0·19 (3)	2·24
	0	Respiration, 62·8±0·1 (12)	4·92±0·39 (6)	1·19±0·09 (4)	2·18±0·09 (6)	2·47±0·05 (6)	3·37
	0·05	Respiration, 118±5·4 (8)	4·07±0·13 (4)	0·33 (2)	1·85±0·19 (4)	—	2·18
	0·20	Respiration, 56±5·7 (8)	5·34±0·14 (4)	0·35±0·09 (4)	0·73±0·06 (4)	—	1·08
	0	Anaerobic glycolysis, 40±1·3 (8)	6·38±0·40 (4)	0·24 (2)	1·05±0·33 (4)	1·53±0·29 (4)	1·29
Glutamate	0	(After cutting, not incubated)	10·12±0·64 (3)	0·35	1·27±0·14 (3)	2·10 (2)	1·62
	0	Respiration, 65·9±1·5 (10)	7·63±0·20 (5)	0·30±0·09 (4)	1·06±0·07 (5)	1·42±0·15 (5)	1·36

measurement even after the dihydroxyacetonephosphate formed has completely reacted. With extracts from slices maintained in glutamate saline it was found that the rate of this extraneous oxidation of DPNH was considerably faster, so that the extrapolation to zero time would give rise to greater error than in the case of other extracts.

The CP values determined by both methods of analysis were consistent and were in close agreement with those reported previously (McIlwain *et al.* 1951; McIlwain & Gore, 1951). Again the sum of the ATP and ADP values could not wholly account for the adenosinepolyphosphate value obtained by the chemical method. Consequently, the sum of the CP and adenosinepolyphosphate values obtained by the chemical method was greater than the corresponding ~P.

It would seem from Table 4 that the adenosinepolyphosphate concentration is maintained at a steadier level than that of CP under adverse conditions. In the presence of 0·05 mM-2:4-dinitrophenol, the CP of the slice is not maintained, but there is only a small decrease in the adenosinepolyphosphate. At a higher concentration of the dinitrophenol (0·2 mM), when the stimulatory effect on respiration disappears, the adenosinepolyphosphate level falls by more than 50%. Significant decreases in adenosinepolyphosphate are observed also in slices maintained under anaerobic conditions in glucose saline and in slices respiring in the presence of glutamate as substrate. Slices tend to lose adenosinepolyphosphate even in the first few minutes after cutting if they are kept in a medium containing glutamate instead of glucose. The values for phosphate liberated on hydrolysis (30 min. in N-HCl) of the calcium-precipitable fraction deserve comment. These 30 min. values tend to be considerably higher than the adenosinepolyphosphate values. The difference between the 10 and 30 min. values may be considered to be a

measure of some phosphate compound other than ATP or ADP which is present in the calcium-precipitable fraction and the variations in its level are interesting. The content of this phosphate compound seems to increase when the adenosinepolyphosphate content decreases, as in slices after cutting, and resynthesis of adenosinepolyphosphate is accompanied by a fall in the level of this compound. From the work of Stone (1943), the hexosemonophosphates may be expected to be present in this fraction, as well as hexosediphosphate. Glucose-6-phosphate, fructose-6-phosphate and hexosediphosphate are all known to release varying proportions of their phosphate on prolonged hydrolysis under the conditions used in this study (LePage, 1949), but since the maximum upper limit for their total concentration in brain slices is the 'blank' value obtained in the enzymic determinations, which is quite low, the much larger differences between the 10 and 30 min. values cannot be entirely accounted for by the presence of hexosemono- and di-phosphates.

DISCUSSION

Because of the known specificity of the hexokinase and myokinase reactions, it may be expected that the enzymic method described in this paper will provide a truer measure of ATP and ADP than methods involving chemical separation followed by hydrolysis of the labile phosphate group. Slater (1953) has assessed the sensitivity of these enzymes in relation to the accuracy of the enzymic determinations based on them. The measurement of CP by the method described here is liable to a greater degree of error since it involves two separate measurements and because of the possible presence of interfering substances for which no correction could be applied. That the error caused by these factors is negligible in cerebral preparations is shown by the agreement between CP values obtained by this method and those obtained by chemical methods. The criticism of the chemical method on the basis of non-specificity for adenosinepolyphosphate is less serious in the case of CP. Since CP appears to be the only substrate in mammalian tissue extracts with a soluble calcium salt and extreme instability in acid molybdate, the chemical method is adequate for its estimation.

Similar considerations of nonspecificity apply also to the measurement of the individual hexosephosphates by methods of chemical separation and differential hydrolysis. While the present method does not distinguish between these compounds, it does give an upper limit for their total content in tissue and gives values considerably lower than those previously reported.

In various circumstances enzymic methods for the determination of ATP were previously reported to give lower values than those obtained by acid hydrolysis (see Bailey, 1949; Kornberg, 1950; Rowles & Stocken, 1950). Consequently, calculations of the distribution of ATP and ADP based on acid-hydrolysable phosphate and total adenine or ribose are subject to considerable error. The errors due to the presence of non-nucleotide phosphates are magnified in the results of such calculations. This is borne out by our finding that in rapidly fixed brain and in cerebral slices kept under conditions capable of supporting phosphate resynthesis, there was no significant amount of ADP. Previous workers using chemical methods of separation have reported considerable quantities of ADP (up to 1·1 μmoles/g.) in rapidly fixed brain (LePage, 1946; Klein & Olsen, 1947; Bain & Klein, 1949). Klein & Olsen (1947) have found changes in the ADP content of brain in varying physiological conditions. Attempts to interpret changes in ADP by an equilibrium constant of creatinephosphokinase as do these authors, do not seem to be justified in view of the unreliability of methods of measuring the nucleotides. Our results with slices also do not support the idea that there is a direct relation between changes in CP, ADP and ATP. While the reserves of CP are rapidly depleted under unfavourable metabolic conditions, total adenosinepolyphosphate does not decrease to the same relative extent under the same conditions.

The changes observed in the labile phosphates of cerebral cortex slices respiring under various conditions are consistent with the dependence of the brain *in vivo* on a steady supply of oxygen and glucose for normal functional activity. The sequence of changes in phosphocreatine and the adenosinepolyphosphates follow a significant pattern. Both with 0·05 mM-2:4-dinitrophenol, and in slices kept at room temperature, it is the phosphocreatine that disappears first. Once the phosphocreatine is lost, ATP tends to fall and concomitantly with this ADP appears in detectable amounts in the tissue. This supports the opinion that the order of utilization of the phosphates on initiation of metabolic activity may be as follows: ATP is first utilized but, under normal conditions, the resulting ADP is rapidly rephosphorylated to ATP by the creatine-phosphokinase system utilizing the phosphocreatine reserves of the tissue. When the phosphocreatine concentration falls below a minimum level, this mechanism is no longer effective, and any further utilization of ATP results in a decrease in the total adenosinepolyphosphate content, and the appearance of ADP in the tissue.

These changes in tissue phosphates may be compared with concomitant changes in tissue respiration. Comparison previously made by McIlwain & Gore (1951) and McIlwain (1952*b*) suggested respiration (and glycolysis) to be conditioned in

part by the levels of inorganic phosphate and of phosphate acceptors in the tissue. The present results with 0·05 mM-2:4-dinitrophenol show increased respiration with little change in any component measured other than creatinephosphate. Creatine may therefore be important in such control. 0·2 mM-2:4-Dinitrophenol may inhibit respiration by its great reduction in energy-rich phosphates, possibly to a level too low for phosphorylation of glucose. Inorganic phosphate, as previously concluded (McIlwain, 1952*b*), cannot alone control respiratory level.

SUMMARY

1. The spectrophotometric method of phosphate analysis developed by Slater (1953) has been adapted for the determination of energy-rich phosphates in mammalian cerebral tissues. It is based on the enzymic formation of dihydroxyacetonephosphate and its enzymic reduction by reduced diphosphopyridine nucleotide.

2. Assays by this method gave values for creatinephosphate which were in close agreement with those obtained by a chemical method.

3. The adenosinediphosphate and adenosinetriphosphate content of cerebral preparations determined enzymically were less than those determined by the chemical method. Only traces of adenosinediphosphate could be found in cerebral cortex *in vivo* or in slices respiring in a glucose-containing saline.

4. Creatinephosphate, adenosinepolyphosphate and inorganic phosphate have been measured in cerebral cortex slices maintained under a variety of metabolic conditions.

We wish to thank Dr E. C. Slater for making his method of determination available to us while still unpublished, as well as for his help throughout this work, and Dr H. McIlwain for his advice and criticism. Acknowledgement is also made to the Australian National University (C. C. Kratzing) and to the British Council (A. Narayanaswami) for the award of scholarships held by the authors during this work.

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On the Isolation and Properties of a Citrate Oxidation Factor from Yeast

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Recent experiments (Foulkes, 1951*a*, *b*) provided evidence for two distinct pathways of metabolism in cell-free preparations from baker's yeast. Pathway 1 is closely related to the tricarboxylic acid cycle normally found in mammalian tissues; all the citrate metabolized under suitable conditions by dialysed, unfortified preparations, can be accounted for by the formation of α-ketoglutaric acid. The addition to such dialysed preparations of magnesium, adenosinetriphosphate (ATP) and a heat-stable organic fraction from yeast or various vertebrate tissues causes a large increase in the amount of citrate metabolized. The heat-stable factor involved in this reaction has been called the citrate oxidation factor (COF) (Foulkes, 1951*b*). The reaction or reactions catalysed by COF will be referred to as Pathway 2.

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The present communication deals with the concentration and partial characterization of COF. Pathway 2, an oxidative decarboxylation of citrate different from the normal tricarboxylic acid cycle, will be described in a subsequent publication.

At this stage, though considerable purification of COF has already been achieved, no final conclusion can yet be drawn on the nature of COF. This is especially so as the percentage of inactive impurities in the final product is difficult to estimate. The possibility must therefore remain that even the purest preparations contain only traces of a very active cofactor.

EXPERIMENTAL

Assay of citrate oxidation factor

In the absence of a stable reproducible assay system or of any knowledge of the chemical properties of COF, no absolute quantitative assay of the latter was possible. To obtain comparative quantitative information it was necessary to add graded dilutions of the fractions to be assayed to the same enzyme preparation. Quantitative data thus obtained could be consistently reproduced. The assumption underlying this assay was that, by analogy with other enzyme-coenzyme systems, a maximum response would be obtained for all concentrations of COF sufficient to saturate the enzyme system. That this assumption was justified can only definitely be proved when purified COF becomes available.

The interpretation of such comparative assays on less pure fractions is rendered difficult by the presence of inhibiting substances. Indeed at some of the earlier steps in the isolation, not only the activity/dry weight, but also the total activity often increases. This finding makes it impossible to attach definite values to the recovery of activity at the various stages. Approximate yields are expressed in terms of 'optimum doses', i.e. the minimum amount of a certain fraction which elicits maximum response in the assay system. It is realized that, because of differences in various enzyme preparations, the 'optimum dose' content can be no more than approximate. In view of this uncertainty no attempts have been made to estimate the final purity by determining activity/dry weight ratios.

The preparation of the cell-free enzyme from yeast has been previously described (Foulkes, 1951*a*). It is dialysed for 4–4·5 hr. in the refrigerator in a rocking dialyser against 0·1 M-phosphate, pH 7·1. The period of dialysis is rather critical: fairly complete removal of COF is not achieved under the present conditions in less than 3–4 hr., whilst irreversible inactivation of the enzyme sets in if dialysis is prolonged much over 5 hr.

To 1 ml. of this dialysed enzyme are added 10 μmoles citrate ($Na_3C_6H_5O_7.2H_2O$, British Drug Houses Ltd., A.R.), 0·1 ml. of 0·85% (w/v) $MgCl_2.6H_2O$, 1 mg. ATP (Boots Pure Drug Co., Nottingham) as the Na salt and the fraction to be assayed, or water; final vol., 2·2 ml. Incubation and analysis are carried out as previously described (Foulkes, 1951*a*). Assays on duplicate flasks should not differ by more than $\pm 5\%$.

The fraction to be assayed must be neutral and free of organic solvents. These may be removed either by distillation *in vacuo* or by exhaustive extraction with ether.

Properties of citrate oxidation factor

In the course of prolonged efforts to isolate COF, over seventy preparations were carried out involving a total of 0·5 ton of yeast. Of the many different purification procedures tested, the one finally recommended is given in the next section; here are outlined some chemical properties of COF as deduced from its behaviour during a variety of methods of isolation.

Stability of citrate oxidation factor. At room temperature COF is stable in a pH range 2–11 for several hours. Normal acid or alkali at higher temperatures caused rapid and complete inactivation. In the frozen state neutral solutions of purified COF have been kept for several months without great loss of activity, although COF appears generally less stable in the purified state than when crude.

Reaction with heavy metals. COF is not precipitated by basic lead acetate, Lohmann's reagent ($Hg(NO_3)_2$ in HNO_3), or Ba^{2+} (in the presence of 90% ethanol at pH 8·4).

Reaction with base precipitants. COF is completely precipitated at pH 1·5–2 by phosphotungstic acid. The recovery of activity from the precipitate is difficult. There is no precipitate formation on addition of picric or picrolonic acid to even relatively concentrated solutions of COF.

Solubility in organic solvents. COF is insoluble in ether. It is precipitated from crude solutions on the addition of 3 vol. acetone. Acetone precipitation from purified solutions leads to considerable inactivation. It is soluble at all pH values in 90% ethanol. In the higher alcohols (*n*-propanol, *n*-butanol) it is more soluble at alkaline than at neutral or acid pH, an observation which has been made use of for chromatographic separation on cellulose columns. From aqueous, salt-saturated solution COF is extracted quantitatively by phenol. Over 90% of the activity is recovered in the combined aqueous phase on washing the phenol layer three times with an equal volume of water.

Use of adsorbents. At pH 3·5, COF is adsorbed by fuller's earth and acid-washed Norite, but not by cellulose (Whatman no. 4 filter paper). Elution is conveniently carried out with dilute ammonia (0·1%).

Behaviour on ion-exchange resins. (*a*) *Basic resins.* COF is not taken up from purified solution by the strongly basic Amberlite resin IR 400. To avoid an undue rise in pH the carbonate form of this resin is to be preferred. There is no interaction between COF and the weakly basic Amberlite resin IR 4 B. (*b*) *Acidic resins.* COF is irreversibly inactivated by the weakly acidic Amberlite resin IRC 50. At pH 3·5, COF reacts with the strongly acidic Amberlite resin IR 100. It can be eluted slowly at pH 3·5, more rapidly at higher pH values.

Chromatography on Amberlite IR 100 has been included in the final purification procedure (step *E*), details of which are given in the next section. Fig. 1 illustrates in detail a typical experiment.

A fraction (25 ml.) purified up to step *D* (see below) was acidified to pH 3·5 with HCl, and applied to columns (int. diam. 1·5 cm., length 35 cm.) containing the resin ground and sieved to 200–500 mesh size. The column had been equilibrated with 0·05 M-sodium citrate, pH 3·5, in 0·05 M-NaCl. Elution was carried out with the same solution. Rate of passage of eluent was 4 ml./hr. It will be seen that the major part of the ultraviolet-absorbing material is eluted before the appearance of COF. This material has been shown to contain uracil and pentose but no phosphate, and it is

hoped to describe it fully elsewhere. In some experiments, due presumably to the effect of uncontrolled variables such as temperature, or to differences in concentration, there may be a greater overlap between the uracil-containing compound and COF. The second lower absorption peak bears no obvious relation to COF and it is not apparent in all experiments.

Reaction with HNO_2. The activity and basic properties of COF are not affected by prolonged treatment with $NaNO_2$ and acetic acid under the conditions employed by Schlenk, Hellström & v. Euler (1938) for the deamination of diphosphopyridine nucleotide (DPN).

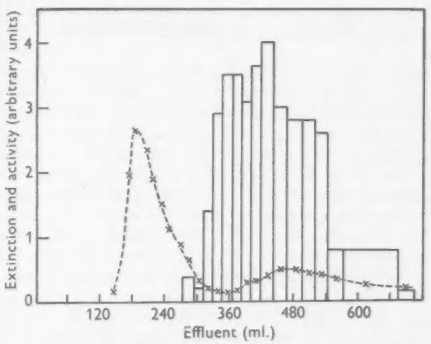

Fig. 1. Behaviour of citrate oxidation factor on Amberlite IR 100. For experimental details, see text. The curve represents extinction at 265 mμ. The superimposed histogram gives the COF activity in arbitrary units.

Properties of aqueous solutions of citrate oxidation factor purified to stage F (see below, p. 327). Such solutions are colourless. There is no specific absorption in the ultraviolet. The ninhydrin test with the sensitive reagent of Moore & Stein (1948) is positive, but can be rendered very weak or absent by treatment with HNO_2 without affecting the activity of COF. It contains no organic or inorganic phosphate, as estimated by the method of Berenblum & Chain (1938) after digestion and hydrolysis in the usual way. There are no reducing sugars before or after 10 min. boiling in 0·3 N-HCl, as estimated by the arsenomolybdate method of Nelson (1944). The reaction with conc. HNO_3 (xanthoproteic reaction) is very weak or absent; the Sakaguchi reaction and biuret test are negative. Only traces of pentose are present as estimated by the orcinol reaction after preliminary bromination to weaken base-sugar bonds (Massart & Hoste, 1947). A fluorescent compound is formed under the conditions of the acetone and alkali reaction for quaternary nicotinamides (Huff & Perlzweig, 1946). However, no evidence could be obtained for the presence of a quaternary nicotinamide by the spectrophotometric cyanide method of Colowick, Kaplan & Ciotti (1951). Drying solutions of purified COF *in vacuo* leads to complete and irreversible inactivation. This occurs also on drying in the presence of dilute NH_3, acetic acid, 2:3-dimercaptopropanol or cysteine. The distillate collected in the liquid air-cooled trap has no activity. There is thus no evidence to indicate that COF is volatile, especially as crude extracts can safely be taken to dryness.

Concentration of citrate oxidation factor

The procedure finally adopted is outlined in Fig. 2 and has yielded consistently good results on a scale ranging from 1 lb. to several cwt. of yeast. The source material is commercial baker's yeast purchased from the Distillers Company Ltd. The values given refer to the preparation from 1 cwt. (51 kg.).

That the overall yield of activity represents only a small part of the COF originally present in the yeast can be deduced from the fact that boiled yeast extract, freshly prepared on a small scale, contains, as far as can be judged, of the order of one 'optimum dose'/2 g. yeast. Each single isolation step recommended below gives satisfactory yields of activity under suitable conditions. Since in large-scale preparations the major losses appear to occur during the initial extraction, this step should be completed as rapidly as possible.

The purification procedure is as follows.

(*A*) One cwt. (51 kg.) of fresh crumbled yeast is extracted with 50 l. tap water. The water is heated to 85° and the yeast added at such a rate that the temperature remains between 85 and 88°. Efficient stirring is essential. When addition is completed, the suspension is heated to 90–92°, then cooled as rapidly as possible below 70°. The faster the original rate of heating, the shorter the time during which COF is exposed to high temperatures, and thus presumably the higher the yield. The cooled suspension is filtered or centrifuged. The final volume is of the order of 55–60 l. No attempt has been made to subject the residue to a second extraction.

(*B*) The filtrate is reduced *in vacuo* to about 6 l. The temperature should not exceed 45°. The pH of the resulting brown syrup is about 5. By the careful addition of 40% KOH the pH is brought to 8·5–9, i.e. just alkaline to phenolphthalein. 600 g. $BaCl_2.2H_2O$ are added and the suspension stirred vigorously. The amount of $BaCl_2$ used probably represents an excess, but because of the copious precipitate formed it is difficult to see whether any $BaCl_2$ remains undissolved. The pH is again adjusted to 8·5. With constant stirring 4 vol. ethanol are added. This concentration in particular is not critical so that the ethanol used may be 95 or 99%. The precipitate is allowed to settle at 0° overnight. There will then be a very heavy, gummy precipitate, and a fairly clear, yellow supernatant. This is poured off and filtered. The most convenient way of recovering the large amount of solution imprisoned in the solid residue is to knead by hand in a few changes of 80% ethanol. Two treatments with 2 l. each should be sufficient. Each time the yellow solution is poured off and filtered. The combined filtrates are then evaporated *in vacuo* to 2 l. As the next step involves extraction of COF into phenol, it is essential at this stage to remove the last traces of ethanol. For this purpose, about 0·5 l. distilled water is added before finally reducing the volume to about 1·5 l. (The Ba precipitate has been used as a convenient source of coenzyme A.)

(*C*) The ethanol-free concentrate is brown in colour, and syrupy in consistency. It contains $BaCl_2$ which would render difficult a determination of the point where sufficient $(NH_4)_2SO_4$ has been added to saturate the solution. An excess of $(NH_4)_2SO_4$ (70 g./100 ml.) is therefore added to the solution at room temperature. The saturated salt solution and sludge is now extracted with repeated amounts of 50–100 g. phenol (A.R.). The extraction is continued till most of the colour has been recovered in the combined

phenolic layers. If the phenolic layer becomes too concentrated at the early stage of the extraction, it will not separate cleanly from the aqueous solution and has to be diluted by the addition of further phenol. It is essential to use a separating funnel with a sufficiently wide bore tap to allow the excess $(NH_4)_2SO_4$ to be withdrawn. In a typical preparation about 400 g. of phenol were used, giving a dark brown phenolic solution of 600 ml. To remove as much of the suspended aqueous layer as possible, the phenol is clarified by spinning and carefully poured off from the salt and salt solution.

$(NH_4)_2SO_4$ carried through the phenol extraction into the final aqueous solution by repeating the $(NH_4)_2SO_4$-phenol-water treatment. The final volume of light brown, aqueous concentrate should be of the order of 200 ml./cwt. yeast.

(*E*) The 200 ml. concentrate is carefully acidified with conc. HCl to pH 3·5. It is then divided into two portions and applied separately to ion-exchange columns. Increasing to any large extent the total material applied to each column leads to overloading and consequent leakage of activity. The columns (int. diam. 2·3 cm.) are tapered at one end and fitted with a coarse sintered-glass filter and tap to

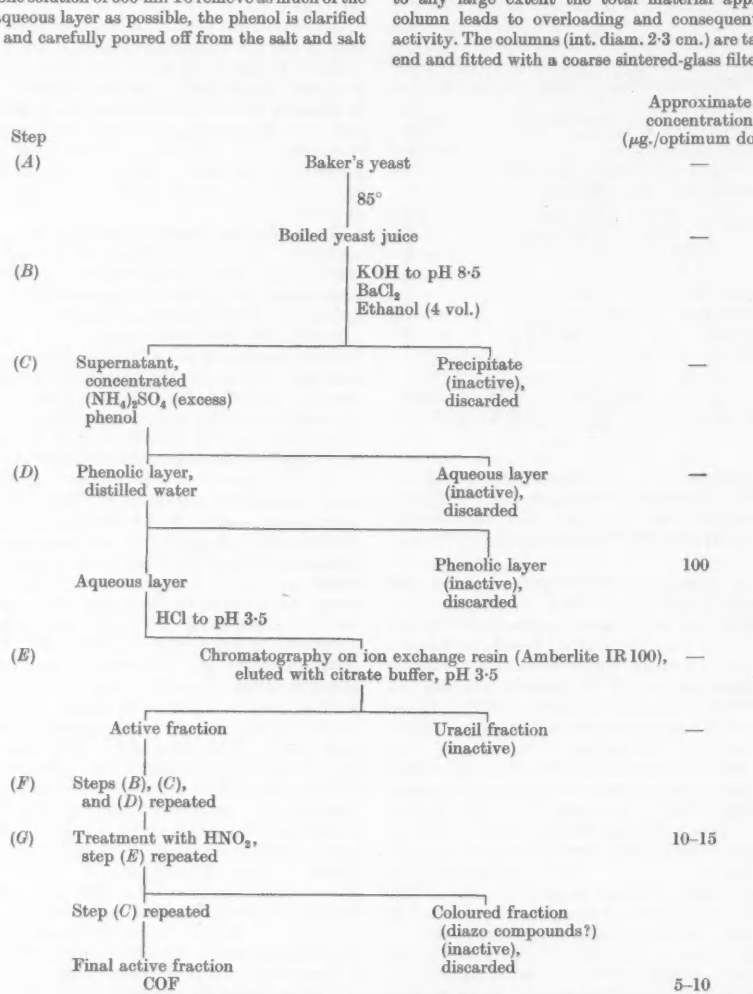

Fig. 2. Purification of citrate oxidation factor.

(*D*) The phenol is extracted three times with 1 vol. each of water, allowing sufficient time for the layers to separate on each occasion. The combined aqueous extracts are freed from phenol by extraction with ether, and evaporated *in vacuo* to about 200 ml. Especially in large-scale preparations it may be desirable to reduce the high concentration of

control the rate of flow. Amberlite IR 100 resin, 200–500 mesh/in., is suspended in dilute HCl and poured into the column to a final height of solid of 48 cm., after removal of excessively fine particles by flotation.

About 100 ml. of the acidified concentrate are applied at room temperature to the column previously equilibrated

with citrate buffer pH 3·5 (10·5 g. citric acid monohydrate, 50 ml. N-HCl, 100 ml. N-NaOH, made up to 1 l.), and elution is carried out with this buffer. A constant head of liquid is maintained over the resin. Collection of fractions is begun when the peak of ultraviolet-absorbing material has appeared in the column effluent. The total volume collected from each column is of the order of 1·5 l. For assay purposes, it is sufficient to neutralize a suitable portion of the citrate buffer and add Mg, ATP and enzyme.

The total time required for this step is about 1 week. The rate of passage of liquid cannot be increased if a good separation is to be achieved. Although contamination of the top of the column by fungi has been observed at the later stages of the operation, no visible growth has ever been found to extend into the column, and the separation of COF is not affected.

(*F*) The combined active fractions (about 3 l.) are neutralized to pH 7 with 40% NaOH. The volume is then reduced *in vacuo* to 300 ml. A high concentration of citrate interferes with the phenol extraction. To repeat the ethanol-Ba fractionation for removal of citrate, alkali (40% NaOH) to pH 8·5, $BaCl_2.2H_2O$ (12·5 g./100 ml.) and 4 vol. ethanol are added as before. It is sufficient to allow the solution to stand at room temperature for 1 hr. before filtering off the barium citrate. This is washed with a few small volumes of 80% ethanol and the combined filtrates are evaporated *in vacuo* to 150 ml., taking the same precautions as previously to remove the last traces of ethanol. The faintly yellow, clear solution is saturated with $(NH_4)_2SO_4$ (75 g./100 ml.) and extracted three times with about 10 g. phenol. The combined phenol layers are clarified by spinning and extracted three times with an equal volume of water. Traces of phenol are removed from the pooled aqueous layer by ether extraction. This leaves 80–100 ml. of a clear, colourless, highly active solution, of which 0·02–0·05 ml. is required for an assay.

(*G*) Further purification can conveniently be achieved by treating this solution with HNO_2, as described above, and rechromatographing it on a small column of Amberlite IR 100 at pH 3·5 as before. The yield of organic material/lb. fresh yeast, averages about 0·2 mg., of which 5–10 μg. are required to elicit an optimum response under the present conditions.

DISCUSSION

In spite of considerable information gained on the chemical properties of COF it is not yet possible to identify it with any compound, or assign to it any particular structure. The following substances were tested for COF-like activity before the information provided by purified preparations narrowed the range of possible structures to weakly basic, phosphate-free compounds: nicotinamide, nicotinamide riboside (given by Dr T. P. Singer), diphosphopyridine nucleotide (40% pure), triphosphopyridine nucleotide (crude), coenzyme III (Singer & Kearney, 1952), biotin (Ashe Laboratories, Leatherhead, Surrey), spermine (given by Prof. Peters), thiamine, cytosine, adenine, cytidine (this and the following five substances were given by Dr D. D. Woods and Dr R. H. Nimmo-Smith), uridine, adenosine, guanosine, cytidylic acid and adenosine-5′-phosphate, and coenzyme A from liver preparations. None of these compounds possesses any activity in the yeast assay system with the exception of the preparation of coenzyme III. This was slightly active, but purified COF has no activity in the coenzyme III-catalysed oxidation of cysteine sulphinic acid (Kearney & Singer, private communication).

The identity of many cofactors with COF can be excluded *a priori* on known chemical data. Among these factors are phosphate-containing compounds such as the cogalactowaldenase of Caputto, Leloir, Cardini & Paladini (1950) or adenosine-5′-phosphate, shown by Kornberg (1951) to be required by a DPN specific *iso*citric dehydrogenase from yeast. Another compound, the chemical properties of which differ greatly from those of COF, is the pyruvate oxidation factor (Gunsalus, Struglia & O'Kane, 1952).

COF has further been tested with negative results for coenzyme A activity in the liver acetylase system of Kaplan & Lipmann (1948). COF has no significant vitamin B activity when assayed after autoclaving with *Streptococcus faecalis* R as test organism (Dr J. Lascelles, private communication). Excluding the identity of various growth factors with COF does not, of course, exclude the possibility that COF is a compound allied to a known factor (conjugated form, breakdown product or precursor).

In its weakly basic properties, acid and alkali lability and resistance to nitrous acid, COF closely resembles nicotinamide derivatives (Schlenk *et al.* 1938). The presence of *N*-substituted nicotinamide is further suggested by a positive Huff & Perlzweig (1946) reaction, but as pointed out above, no spectrophotometric evidence could be obtained to support the view that COF is a nicotinamide derivative.

Whatever structure is finally assigned to COF, it will have to explain the instability of purified fractions under various conditions. It cannot be decided at the moment whether this property is due to presence of damaging impurities (e.g. compounds originating from the resin) or whether it is caused by some intramolecular reaction such as a lactone formation. Further work along these lines is in progress.

SUMMARY

1. A method is described for the partial purification of the citrate oxidation factor (COF). This is a compound required by cell-free yeast extracts for the metabolism of citrate by a pathway apparently outside of, and additive to, the normal tricarboxylic acid cycle.

2. COF is a weakly basic compound, free from phosphorus. Its possible structure is discussed in the light of this and other data.

This work was carried out during the tenure of an Australian National University Scholarship. It was made possible by the constant guidance and encouragement of Prof. Sir Rudolph Peters, F.R.S., to whom I wish to express my sincerest thanks. Dr D. D. Woods, F.R.S., Dr L. A. Stocken, Dr P. W. Kent, Mr R. W. Wakelin and many others greatly assisted by their continuous helpful discussions and advice. The large-scale work would have been impossible without the willing assistance of Mr B. K. Kelly and his staff at the M.R.C. Antibiotics Research Station, Clevedon. I also wish to thank Dr W. E. van Heyningen for allowing me to use his vacuum still. Miss V. Bustin's skilful assistance during a limited period proved most helpful.

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The Effect on Mouse-liver Catalase Activity and Blood-haemoglobin Level of a Milk Diet Deficient in Iron, Copper and Manganese

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Recent work has shown that tumours contain some agent capable of rapidly and markedly depressing liver catalase activity in mice (Nakahara & Fukuoka, 1949, 1950; Fukuoka & Nakahara, 1951; Adams, 1950*a*, 1951*a*; Greenfield & Meister, 1951). Further, the importance of adrenal and testicular hormones in the maintenance of mouse-liver catalase activity was demonstrated by Adams (1950*b*, 1952) and may be briefly summarized as follows. There is a sex difference in enzyme activity, males having the higher level, and castration results in a fall in activity to slightly below the normal female level. Testosterone injected into castrated males or normal females produces a rise in catalase activity to approximately that of normal males. A marked fall in enzyme activity follows adrenalectomy in both sexes, and the level is restored to normal by the injection of cortisone. Evidence was then obtained that the tumour agent acts by blocking the normal hormonal stimulation of the liver (Adams, 1951*b*).

In order to gain more information about the normal mechanisms involved in the maintenance of mouse-liver catalase activity it was decided to examine the role of trace metals. Since catalase is an iron-containing enzyme, it is not unreasonable to suppose that trace metals play an important part in its synthesis, and in fact Schultz & Kuiken (1941) found that rat-liver catalase activity was depressed when the animals were maintained on a diet deficient in iron or copper. Addition of the missing metal restored the activity to normal. Fukuoka & Nakahara (1951) stated that feeding or injecting ferric chloride stimulated liver catalase activity in the tumour-bearing animal. Little or no other work on the influence of trace metals appears to have been done, apart from a report by Day & Skidmore (1947) that catalase activity is depressed in zinc deficiency.

The present work began with an attempt to repeat in mice the effects of iron and copper deficiency noted in rats by Schultz & Kuiken (1941). However, the results with a trace-metal-deficient diet differed so widely from those obtained by these authors that it was found necessary to carry out a more detailed investigation. Accordingly, the effects of deficiencies of iron, copper and manganese, on liver catalase activity have been studied. The degree of iron and copper deficiency was checked by corresponding determinations of haemoglobin level. An attempt was also made to find a connexion between the observed effects and the action of testosterone on catalase level.

EXPERIMENTAL

Diet. Commercially supplied 'homogenized' whole milk supplemented with 3% (w/v) glucose (A.R. quality) was used as the basic diet. A number of milk-glucose samples were analysed for Fe and Cu. The Fe content varied from 0·36 to 0·45 p.p.m., and the Cu content from 0·14 to 0·3 p.p.m. A single Mn estimation on a pooled sample gave a value of 0·03 p.p.m. (Analyses by Dr R. F. Milton.)

Leakage from the drinking bottles was rather troublesome but the mice each consumed 6–8 ml./day. Drinking water was not provided. $MnSO_4$, A.R., $FeSO_4$ (limit of Cu impurity 0·00075%) and $CuSO_4$ (limit of Fe impurity 0·0028%) were added to the basic diet as required, to provide 5 μg. Mn, 50 μg. Fe and 10 μg. Cu/mouse/day, based on the above consumption figures. As far as could be estimated, during the first 4 or 5 weeks on milk the intake per mouse was largely independent of trace-metal addition. In the later stages, mice on a diet of milk supplemented with Fe, Cu and Mn increased their intake appreciably. Since, however, the important changes to be described occurred during the first 5 weeks, no attempt at paired feeding was made.

Although milk is not a particularly suitable diet (largely because of its liquid nature and low total-solid content), it has been extensively used in trace-metal deficiency studies. Milk supplemented with Fe, Cu and Mn was stated to support good growth to maturity in rats (Kemmerer, Elvehjem, Hart & Fargo, 1932). Ideally, milk should be collected directly from the cow into glass vessels, but owing to the practical difficulties of obtaining regular supplies in this way, the present investigation has been made using 'quick pasteurized' milk provided commercially. One advantage of this procedure is that homogenized milk in which no separation of the cream occurs is readily available. Homogenized milk + 3% (w/v) glucose, supplemented with Fe, Cu and Mn, has in fact given steady growth over periods of up to 7 weeks (the duration of the longest experiment). There have been no obvious signs of thiamine deficiency. It is true that a cube diet supplied *ad lib.* produces a more rapid growth rate (Table 1) but this may well be due to overeating, since on this diet the animals tend to deposit large masses of body fat after a few weeks. The normal diet used in this work consisted of cubes prepared according to the Rowett Institute formula (Thomson, 1936).

The following abbreviations will be used in referring to the various milk diets: milk + 3% (w/v) glucose without trace metal supplements—milk; milk + 3% (w/v) glucose supplemented with Fe—milk-Fe; with Cu—milk-Cu; with Mn—milk-Mn; with Fe + Cu—milk-FeCu; with Fe + Mn—milk-FeMn; with Cu + Mn—milk-CuMn; with Fe + Cu + Mn—milk-FeCuMn. The amounts of the supplements have been given below.

The mice were kept in heavily galvanized cages, and on heavily galvanized grids which prevented access to the sawdust. These conditions are by no means ideal, but in practice a rapid anaemia could be produced, even with a diet deficient only in Fe.

Animals. Weanling mice (wt. 10–12 g.) of a stock albino strain were used.

Estimation of haemoglobin and liver catalase activity. Blood taken from the neck immediately after decapitation was pipetted into 0·1 N-NaOH, and at least 30 min. allowed for full colour development. The haemoglobin level was then read from a calibrated colorimeter.

Samples of whole liver disintegrated in a homogenizer were allowed to react with 0·025 M-H_2O_2 (A.R.) in 0·2 M phosphate buffer (pH 6·8) at 0°, the reaction being stopped after 4 min. by the addition of H_2SO_4. The H_2O_2 remaining was estimated by titration with standard $Na_2S_2O_3$ after the addition of excess KI. Corresponding determinations of total N were made (Kjeldahl), catalase levels are expressed in arbitrary units/mg. N. For details, and reason for the use of N as a standard, see Adams (1950*a*, 1952).

Operative procedure. Castration was carried out under ether-$CHCl_3$ anaesthesia as previously described (Adams, 1952).

Testosterone. (Commercially supplied for implantation.) It was injected intraperitoneally in 50% (v/v) ethanol-glass-distilled water solution. The ethanol used was redistilled in glass apparatus cleaned with glass-distilled water.

RESULTS

Fig. 1. shows the development of liver catalase activity in weanling mice on a normal cube diet. At the weaning stage the catalase activity was only

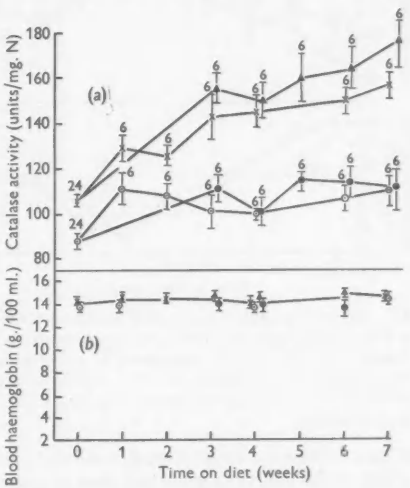

Fig. 1. (*a*) Development of liver catalase activity in weanling albino mice maintained on a normal cube diet (males, ×—×; females, ⊙—⊙) and on milk supplemented with iron, copper and manganese (males, ▲—▲; females, ●—●). (*b*) Some corresponding haemoglobin levels. In this and subsequent figures results are given as arithmetic means ± standard error of means. The number of animals in each group is given at the head of the catalase-standard-error limits.

slightly higher in males than in females, and in both was below adult level. The sex difference in catalase activity became fully developed about 3 weeks after weaning, due to a rapid increase in the male level. The mice were then 5–6 weeks old. Little subsequent change in activity occurred, although there

was a further slight rise in the male level during the next 3 weeks. Fig. 1 also shows the effect of maintaining mice on the milk-FeCuMn diet. The catalase activity in both sexes developed in the same way as on the cube diet, the only difference in the two sets of curves being a tendency for the supplemented-milk animals to maintain a slightly higher level. In both the supplemented-milk and the cube diets the haemoglobin level remained practically unchanged. The results contrasted sharply with those obtained in mice kept on a diet of milk alone (Fig. 2). There was little change in either the male or female catalase level during the first 2 weeks, followed by a sharp rise in activity in both sexes,

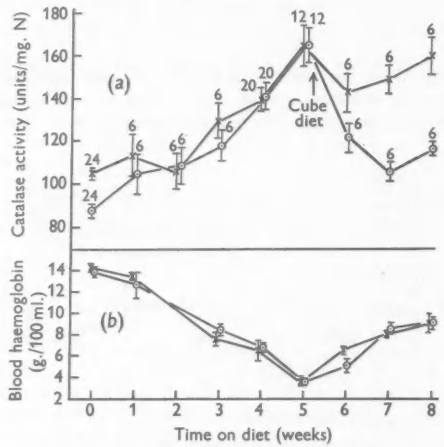

Fig. 2. (*a*) Development of mouse-liver catalase activity on a diet of milk without trace metal supplements. After 5 weeks the animals were transferred to a normal cube diet (males, ×—×; females, ⊙—⊙). (*b*) Corresponding haemoglobin levels. Figures refer to number of animals in each group.

the sex difference disappearing entirely. After 5 weeks the level in both sexes had risen to approximately the normal male level, while the blood haemoglobin had fallen to just below 4 %. At this stage the animals had cream-coloured livers, and large bald areas of skin. Body weight had increased by only about 30 % above the initial value (Table 1) compared with about 150 % in mice on normal diet. If the animals were kept on the milk diet beyond this stage, death ensued rapidly. However, they recovered if transferred to a normal cube diet after 5 weeks on milk, and Fig. 2 also shows the effect of this on catalase and haemoglobin levels. Although male catalase activity remained more or less unchanged, the female level fell sharply towards normal. The haemoglobin level rose, but more slowly, reaching 9 g./100 ml. after 3 weeks. Restora-

Table 1. *Mouse body weights at varying times when maintained on normal cube diet, milk, or milk supplemented with iron, copper and manganese*

(Results are given in g. as arithmetic means ± standard error of means. Mice weighed to the nearest g. The number of animals in the groups are given by the figures in brackets.)

Diet	Sex	Controls	Time on diet (weeks) 1	2	3	4	5	6	7	8
Milk	M.	↑	10 ± 1·0 (6)	15 ± 0·7 (6)	15 ± 1·1 (6)	16 ± 0·5 (20)	16 ± 0·6 (12)	23 ± 0·4 (6)	26 ± 1·3 (6)	30 ± 0·4 (6)
	F.		9 ± 0·9 (6)	16 ± 1·1 (6)	18 ± 1·1 (6)	18 ± 0·4 (20)	15 ± 1·0 (12)	22 ± 1·3 (6)	24 ± 0·9 (6)	29 ± 0·7 (6)
Cube	M.	12 ± 0·3 (24)	14 ± 0·4 (6)	19 ± 1·1 (6)	24 ± 1·7 (6)	26 ± 1·0 (6)	30 ± 0·9 (6)	33 ± 1·6 (6)	—	—
	F.	11 ± 0·3 (24)	14 ± 0·5 (6)	20 ± 0·7 (6)	21 ± 1·3 (6)	23 ± 0·8 (6)	26 ± 1·2 (6)	31 ± 1·4 (6)	—	—
Milk supplemented with iron, copper and manganese	M.		—	—	17 ± 0·1 (6)	20 ± 1·1 (6)	25 ± 1·2 (6)	28 ± 1·6 (6)	29 ± 0·8 (6)	—
	F.	↓	—	—	16 ± 0·6 (6)	19 ± 0·4 (6)	20 ± 0·6 (6)	25 ± 0·8 (6)	26 ± 1·0 (6)	—

Transferred to normal diet

Table 2. *Liver catalase activity and haemoglobin levels in mice maintained on a diet of milk supplemented with iron, and the effect of addition of copper*

(Activities in arbitrary units/mg. N are given as arithmetic means ± standard error of means. The number of animals in the groups are given by the figures in brackets. Corresponding blood-haemoglobin levels in g./100 ml. as arithmetic means ± standard error of means.)

	Sex	Controls	Time on diet (weeks) 2	3	4		5
Catalase	Male	106 ± 2·6 (24)	102 ± 4·4 (6)	146 ± 7·5 (6)	152 ± 11·0 (6)	Copper added to diet	143 ± 8·0 (7)
Haemoglobin	Male	14·3 ± 0·30	8·6 ± 0·80	5·6 ± 0·68	3·5 ± 0·22	Copper added to diet	7·4 ± 0·69
Catalase	Female	88 ± 3·6 (24)	109 ± 3·4 (12)	138 ± 5·6 (12)	149 ± 5·3 (12)	Copper added to diet	102 ± 5·4 (8)
Haemoglobin	Female	13·8 ± 0·40	8·3 ± 0·72	7·7 ± 0·51	5·4 ± 0·5	Copper added to diet	7·9 ± 0·23

tion of hair began, and the liver darkened to its usual colour. Body weight also increased rapidly, more than doubling itself during the 3-week period (Table 1). Table 1 also shows the development of body weight on the milk-FeCuMn diet. On this diet the hair growth and liver colour were normal.

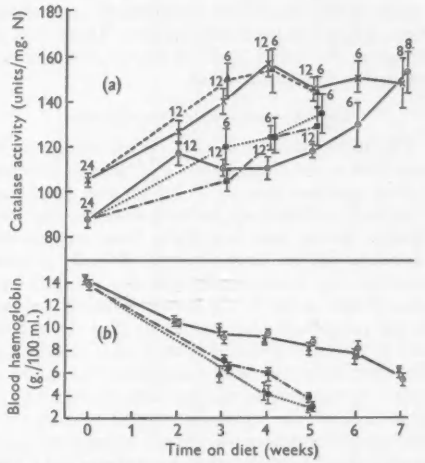

Fig. 3. (*a*) Effect of trace metal supplements on the development of catalase activity in weanling mice kept on a milk diet. Copper (males, ×—×; females, ⊙—⊙); manganese (males, ▲—▲; females, ●···●); Iron + manganese (females only, ■-·-■). (*b*) Corresponding haemoglobin levels. Figures refer to number of animals in each group.

Addition of iron to the milk (milk-Fe) had little effect on catalase or haemoglobin levels as may be seen from Table 2. The sex difference in catalase activity again disappeared, the level in both sexes approximating to that of normal males, and a haemoglobin level of 5 g./100 ml. was reached after 4 weeks; very pale livers, loss of hair, and failure to gain weight were also observed in these mice, although the effects seemed less marked than in the mice kept on milk alone. Copper was then added to the milk-Fe diet of the small group of mice remaining in this experiment. A week later the female enzyme level had fallen to normal, there being little or no change in the males. The haemoglobin in both sexes had risen to nearly 8 g./100 ml.

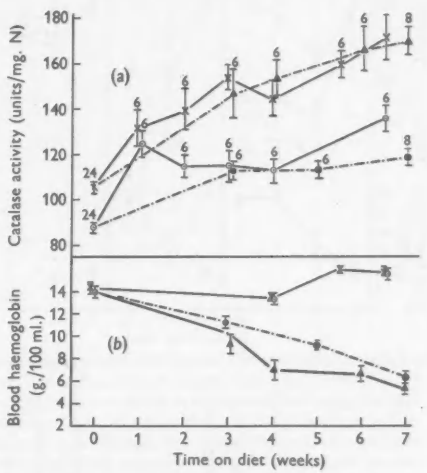

Fig. 4. (*a*) Effect of trace metal supplements on the development of liver catalase activity in weanling mice kept on a milk diet. Iron + copper (males, ×—×; females, ⊙—⊙); copper + manganese (males, ▲-·-▲; females, ●-·-●). (*b*) Some corresponding haemoglobin levels. Figures refer to number of animals in each group.

The sex difference in catalase activity in animals given the milk-Cu diet developed in a normal way for the first 5 weeks (Fig. 3) (Hb down to 8·6 g./100 ml.). Subsequently, however, the female level rose, reaching the male level after 7 weeks on the diet. The milk-Mn diet was less effective in producing a normal picture (Fig. 3), but the sex difference in catalase activity was apparent for 4 weeks, although the female level was rather higher

than normal. Subsequently, the female level rose still higher, and the male level was almost reached by the fifth week. The experiment was then terminated, the haemoglobin having fallen to 3 g./100 ml. Fig. 3 also shows that females on the milk-FeMn diet behave similarly to those on a manganese supplement only.

The effects of feeding the milk-FeCu and milk-CuMn diets are shown in Fig. 4. The curve obtained with milk-FeCu resembled that with milk-Cu (Fig. 3), the female level being normal for 4 or 5 weeks. The female level then rose, and there appeared to be an upward trend in the male level also. In contrast to the results with milk-Cu, the haemoglobin level in mice on the milk-FeCu diet remained normal for the duration of the experiment. The catalase activity developed normally on the milk-CuMn diet, the results resembling those with milk-FeCuMn. Haemoglobin, however, fell to approximately 6 g./100 ml. after 7 weeks on the diet.

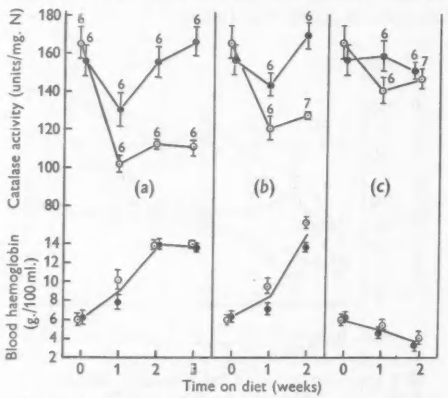

Fig. 5. Variation in liver catalase activity (upper graphs), and haemoglobin level (lower graphs) resulting from the addition of copper and manganese to the diet of mice kept on milk supplemented with iron for 4 weeks. (*a*) Copper and manganese; (*b*) copper only; (*c*) manganese only. Control mice on the left of each graph (the same group being used in each case). (Males, ●—●; females, ⊙—⊙.) Figures refer to number of animals in each group.

Addition of copper and manganese to the diet of deficient mice

A number of male and female mice were kept on the milk-Fe diet for 4 weeks. The catalase activity and haemoglobin level of a small group of each sex were then determined, and the remainder divided into three groups. These groups were given copper only, manganese only, and copper plus manganese, respectively, as supplements to the milk-Fe diet. Catalase and haemoglobin were determined after 7 days, after 14 days, and in the case of the copper and manganese supplement, after 21 days. The results appear in Fig. 5.

The copper and manganese supplement caused a sharp fall in the female catalase activity. The normal level was reached after 1 week, and there was no subsequent change. There was also a fall in the male catalase activity after 1 week, but by the second week the level had risen to its normal value. Haemoglobin was restored to normal after 2 weeks, in both sexes. A comparison with Fig. 2 shows that the result of the addition of copper plus manganese to the milk-Fe diet was closely similar to that obtained when mice which had been kept on milk for 5 weeks were transferred to a cube diet.

The addition of copper only, and manganese only, to the milk-Fe diet was not so successful in restoring a normal catalase activity picture. The copper supplement caused a marked fall in the female catalase activity, and a slight fall in the males, after 1 week. The female level was still rather high, however. After 2 weeks the male level had risen again, and the female level also rose slightly. Haemoglobin returned to normal in 2 weeks. Only a slight fall in the female catalase activity resulted from the manganese supplement. There was no change in the males, and the haemoglobin level in both sexes continued to fall.

Experiments with castrated males

The most striking feature of the results so far described is the disappearance of the sex difference in liver catalase activity in copper and manganese deficiency and its reappearance after feeding these metals. In no case has there been any marked variation in the development of male catalase activity—the disappearance of the sex difference being due to a rise in the female level. It has been shown previously (Adams, 1952) that the sex difference is due to lack of testosterone in the females. However, from the general appearance of male mice after 4–5 weeks on the milk or milk-Fe diets, and the small size of the testes, it seemed reasonable to assume that testosterone was not being produced in normal quantities. The diet experiments were therefore repeated in castrated males. The mice were castrated at weaning age, and the control catalase and haemoglobin levels measured 48 hr. later. Control mice were kept on a cube diet for this 48 hr. period. Table 3 shows the result of keeping castrated males on the cube diet, milk only, and the milk-Fe diet. The results were closely similar to those already obtained with females and normal males under the same conditions, i.e. a sharp rise in activity up to the normal male level. On the cube diet there was a very slight rise in catalase level over a period of 6 weeks. After 4 weeks on milk only, the remaining animals were placed on the cube diet. Catalase activity fell steadily, reaching the normal value for castrated males within 3 weeks. Haemoglobin rose during this period from just below 4 to 11·5 g. %. The last group

Table 3. *Liver catalase activity in castrated male mice maintained on various diets*

(Results are given in arbitrary units/mg. N as arithmetic means ± standard error of means. The number of animals in the groups are given by the numbers in brackets. Corresponding blood-haemoglobin levels in g./100 ml. ± standard error of means. Details of control experiments given in text.)

Diet	Controls		Time on diet (weeks) 2	3	4	5	6	7
Milk	↑	Catalase	110±7·0 (6)	147±11·2 (6)	158±8·1 (6)	Transferred to normal diet: 124±6·6 (6)	104±7·1 (6)	91±3·2 (6)
		Haemoglobin	7·3±0·38	5·9±0·22	3·9±0·26	4·5±0·24	6·4±0·28	11·4±0·53
Milk + iron		Catalase	100±5·6 (6)	152±9·2 (6)	154±6·2 (6)	147±9·9 (6)	Copper added to diet: 116±7·0 (6)	—
		Haemoglobin	12·8±0·22	9·4±0·48	7·5±0·62	6·5±0·38	10·5±0·55	—
Milk + copper	Catalase; 84±2·7 (8)	Catalase	101±5·2 (6)	105±3·9 (12)	113±7·0 (12)	—	133±10·4 (6)	—
		Haemoglobin	12·7±0·66	10·5±0·55	10·0±0·59	—	6·7±0·93	
Milk + iron + copper	Haemoglobin; 13·8±0·5	Catalase	—	—	107±4·7 (6)	127±6·7 (6)	—	148±7·9 (6)
		Haemoglobin	—	—	13·3±0·46	13·4±0·69	—	16·0±0·36
Milk + copper + manganese		Catalase	—	121±6·9 (6)	128±7·3 (6)	—	156±8·1 (8)	—
		Haemoglobin	—	9·3±0·52	8·3±0·51	—	5·8±0·70	—
Milk + iron + copper + manganese		Catalase	—	112±8·6 (6)	103±6·7 (6)	—	116±4·7 (8)	—
		Haemoglobin	—	11·7±0·12	13·0±0·50	—	12·4±0·41	—
Cube diet		Catalase	—	—	95±6·5 (6)	—	102±6·4 (6)	—
	↓	Haemoglobin	—	—	13·5±0·62	—	14·5±0·50	—

Table 4. *A summary of catalase and haemoglobin levels in mice after 4–5 weeks on various diets*

	Normal cube diet	Milk supplemented with: —	Iron	Copper	Manganese	Iron + copper	Iron + manganese	Copper + manganese	Iron + copper + manganese
Normal males									
Catalase	High	High	High	High	High	High	—	High	High
Haemoglobin	Normal	Very low	Low	Medium	Very low	Normal	—	Medium	Normal
Normal females									
Catalase	Low	High	High	Low to medium	Medium	Low to medium	Medium	Low	Low
Haemoglobin	Normal	Very low	Low	Medium	Very low	Normal	Low	Medium	Normal
Castrated males									
Catalase	Low	High	High	Low to medium	—	Low to medium	—	Medium	Low to medium
Haemoglobin	Normal	Very low	Low	Medium	—	Normal	—	Medium	Normal

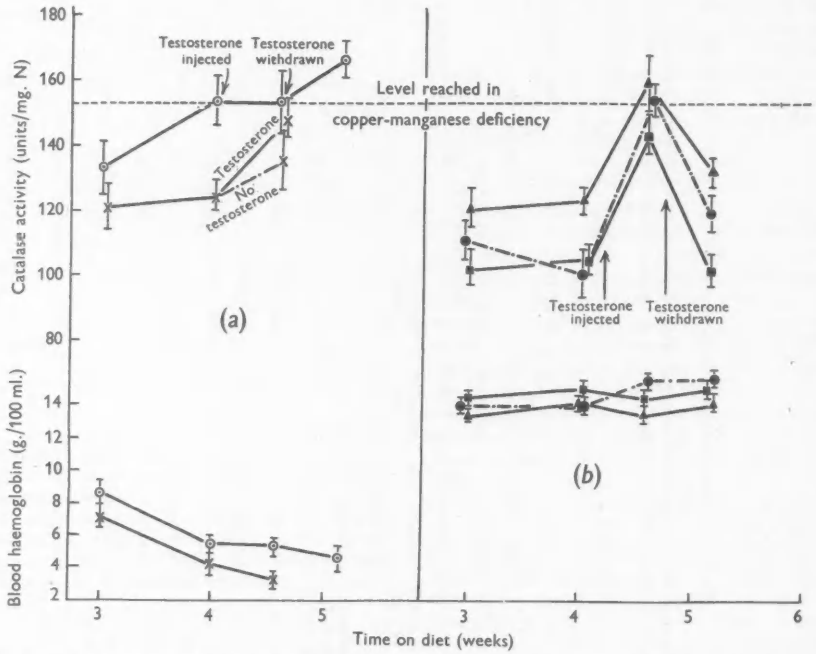

Fig. 6. The effect of testosterone injection and withdrawal on the liver catalase activity (upper graphs) of female mice kept on various diets. Corresponding haemoglobin levels are given in the lower graphs. Six animals per group. (*a*) Milk + iron, ⊙—⊙; milk + manganese, ×—×. (*b*) Normal diet, ■—■; milk + iron + copper, ▲—▲; milk + iron + copper + magnanese, ●-·-·●.

of mice kept on the milk-Fe diet were given copper as well after 5 weeks. One week later the catalase activity had fallen sharply and the haemoglobin had risen to 10·5 g./100 ml.

Table 3 also shows the effect on catalase activity of maintaining castrated mice on the milk-Cu, milk-FeCu, milk-CuMn and milk-FeCuMn diets. The last diet was the most successful in maintaining the low level normal for castrated males, although there was a slight upward trend. Milk-Cu and milk-FeCu were less satisfactory, particularly in the later stage (5–6 weeks on diet). However, all these diets were

markedly superior in this respect to milk alone, or to milk-Fe, for at least the first 4 weeks. The haemoglobin level in these mice was maintained by the milk-FeCu or milk-FeCuMn diets. It was expected that there would be no difference between milk-CuMn and milk-FeCuMn in the maintenance of a normal catalase activity, as had been found in females. However, on the former but not on the latter diet the catalase activity in castrated male mice rose steeply, the normal male level being reached within 6 weeks. Table 4 summarizes the catalase and haemoglobin levels observed after 4–5 weeks on various diets.

The influence of testosterone

It appears that under all the dietary conditions described the catalase activity of normal male mice reached approximately the same point, which lies within the range of normal values for this strain of mice. There was a tendency for the enzyme level to be in the high normal range, but values which certainly exceed normal limits have not been observed. The rises in level in castrated male or normal female mice under conditions of copper and manganese deficiency have been approximately equal to the rises which would be expected in these animals after treatment with testosterone. Thus, copper-manganese deficiency appears to be equivalent to testosterone in its effect on liver-catalase level. But it does not follow that the effects are connected. To gain evidence on this question, testosterone was injected into females maintained on various diets. It was soon found that female mice in the early stages of a milk diet would not tolerate the daily injection of 100–150 μg. of testosterone in 0·03 ml. of 50 % (v/v) ethanol-water. After 4 weeks on the diets the injections were taken well, but the reason for this is not known. Testosterone was therefore injected daily in 150 μg. doses into female mice, which had been maintained for 4 weeks on the cube diet, and on milk-Fe, milk-FeCu, milk-Mn and milk-FeCuMn, respectively. After 4 days, catalase activities were determined, and the testosterone withdrawn from the remaining mice. After a further 4 days the catalase activity of the remaining mice was determined. In the case of the mice receiving milk-Mn, half received testosterone after 4 weeks on the diet and the remainder did not, the catalase activity of both groups being determined after the testosterone had been injected for 4 days. This altered procedure was used because it seemed unlikely that the mice would survive the full length of the experiment. There was no response to testosterone in the mice kept on milk-Fe. A response was obtained in mice kept on the other diets, but in no case did the testosterone injections cause the catalase activity to exceed the level reached in copper-manganese deficiency (Fig. 6). The catalase activity rose to approximately this level, and fell appreciably when the testosterone was withdrawn. The catalase activity of the manganese-supplemented mice injected with testosterone rose slightly above that of the non-injected controls, but only to the same level as in the other groups.

DISCUSSION

This work has shown that a milk diet deficient in iron or copper, or both, produces a considerable degree of anaemia within about 4–5 weeks. This agrees with the earlier results (e.g. Cohn *et al.* 1927) in showing that iron and copper are essential for the synthesis of haemoglobin, while manganese is not.

Milk supplemented with iron, copper and manganese maintained a normal high catalase level in male mice and a normal low level in female and castrated male mice. On unsupplemented milk or milk-Fe the sex difference disappeared, due to a rise in the female level, the male level remaining substantially unchanged, but castrated males behaved in the same way as females. This rise in catalase level in females and castrated males appeared to be due primarily to a copper and manganese deficiency, suggesting that copper and manganese exert a depressant action on catalase synthesis, contrasting with the stimulating action of copper on haemoglobin synthesis. Castrated males fed milk-CuMn did not behave as expected, however; catalase activity was not held at the normal low level. Possibly this result was due to some other factor, such as defective absorption. Nevertheless, catalase levels over the first 4 weeks were considerably lower than in mice on milk or milk-Fe.

The effect of copper and manganese deficiency in mice has been shown to be approximately the same as that of testosterone. Moreover, testosterone treatment failed to produce a rise above the copper-manganese-deficient level. It is, of course, possible that mice maintained on milk-Fe for some weeks are in too poor a condition to react to testosterone at all. Nevertheless, mice on all other diets have shown some response to testosterone, the maximum levels of catalase activity being similar, and approximately equal, to that reached in copper-manganese deficiency. This suggests that testosterone stimulates catalase synthesis in an indirect way; possibly by inhibiting the depressing action of a copper-manganese system. It would be interesting to see whether cortisone acts through a similar mechanism, although the practical difficulties of using the present method would be very great. Survival of copper-deficient adrenalectomized animals would be unlikely. Cortisone and testosterone certainly exert independent effects on catalase

activity (Adams, 1952). A hypothesis that testosterone acts through a copper-manganese system, while cortisone does not, would explain the observed results.

Iron added to milk produced little effect on catalase level either alone or in combination with other metals, although it was required, in addition to copper, for the maintenance of a normal haemoglobin level. As far as catalase is concerned, an iron-deficiency effect seems difficult to produce. Presumably if the iron content of the body could be reduced to a point at which insufficient was available for catalase synthesis the enzyme level must fall. However, this may not be possible. In the seventh week of the experiment on milk supplemented with copper the catalase activity of the females rose from 130 to 155 arbitrary units, whilst the haemoglobin level fell from 8 to 6 g./100 ml., suggesting that liver catalase is synthesized preferentially to haemoglobin.

The general picture differs so markedly from that suggested by the results of Schultz & Kuiken (1941) with rats, that it is hardly possible to offer an explanation of the discrepancy, beyond the obvious suggestion of a species difference. However, these authors did find that heart catalase activity increased on a copper-deficient diet.

SUMMARY

1. In weanling mice a diet of milk and glucose supplemented with iron, copper and manganese is equivalent to a normal cube diet for the normal development of liver catalase activity. There is a marked sex difference in enzyme activity from about 5 weeks of age.

2. The sex difference in catalase activity disappears in mice kept on a diet of milk alone, the enzyme level in both sexes approximating to that of normal males. The haemoglobin level falls sharply during this process. If the mice are transferred to a normal cube diet after 5 weeks on milk, the haemoglobin and female catalase levels return to normal, the male catalase activity remaining unchanged and at its normal high level.

3. Castrated males kept on milk supplemented with iron, copper and manganese, maintain their normal low level of catalase activity. On a milk diet unsupplemented with trace metals, the level rises just as in females to approximately that of non-castrated males.

4. Addition of iron to milk produces no change in the catalase levels reached with milk alone, although haemoglobin usually falls less rapidly.

5. Addition of copper or manganese to milk is partially effective in maintaining a normal development of catalase activity. Copper and manganese together are as effective as iron, copper and manganese in normal females.

6. In animals maintained for 4 weeks on a diet of milk supplemented with iron, the addition of copper, or copper and manganese, produces a sharp fall in the female catalase level, leaving the male level substantially unchanged. Manganese added to milk and iron is less effective, and copper and manganese together are more effective than copper alone in restoring the normal level in females. The addition of copper to the diet of castrated males kept on milk and iron causes a sharp reduction in catalase activity, to near the normal level.

7. Testosterone injected into females maintained on various diets gives a rise in catalase activity only to the level obtained in copper manganese deficiency. No further rise is obtained in females in which the level was already raised by copper and manganese deficiency.

8. Copper- and manganese-deficient mice showed other evidence of abnormality, notably loss of hair, and very light-coloured livers.

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Vitamin D Balance and Distribution in Rats Given a Massive Dose of Ergocalciferol

PART I. EXPERIMENTAL RESULTS

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Since no entirely satisfactory chemical or physical method is yet available for the quantitative estimation of vitamin D in animal tissues, resort must be had to laborious biological assays. Consequently there is little information about the amount and site of storage of the ingested vitamin. Nor is there much experimental evidence to indicate what proportion of an ingested dose can be recovered from tissues and excreta. Windorfer (1938) reported that in rachitic infants receiving 10–15 mg. of ergocalciferol, less than 10% was excreted within 10 days after dosing, and in one instance in which an autopsy was performed less than 10% was found in the tissues. Vollmer (1939) and Houet (1946) found that the tissues of children who had received a massive dose of ergocalciferol contained only a small proportion of the ingested vitamin. Morgan & Shimotori (1943) studied the distribution in the tissues of young dogs 3 days after a massive dose. The vitamin did not seem to be stored in any one organ. The greatest concentrations were found in heart, kidney and intestine, but all tissues contained small amounts, from 1 to 5 i.u./g.

The present paper reports experiments designed to ascertain, in rats given a large dose of ergocalciferol, (*a*) the distribution of the vitamin in tissues and excreta at various time intervals, and (*b*) the percentage of the dose recovered. A preliminary communication has already been presented (Cruickshank & Kodicek, 1952*a*).

EXPERIMENTAL

The studies consisted of one main and two subsidiary experiments. In the former, assays were carried out on freeze-dried tissues and faeces, in the latter on fresh. It was thus possible to assess the effect of freeze-drying on the vitamin D content.

Material

In all experiments the test substances were derived from piebald rats depleted of vitamin D, i.e. rats maintained from weaning on the rachitogenic diet 2965 of Steenbock for about 3 weeks, by which time florid rickets had developed. They were then given ergocalciferol *per os*, the same diet being continued. Material from deficient rats which did not receive the vitamin was also assayed.

In the main experiment (Exp. 1) the rats were divided into five groups, each comprising five rats. Each rat in four of the groups received a single dose of 1 mg. ergocalciferol (40000 i.u.) in arachis oil. The rats in the remaining group were given arachis oil only, and served as controls. The design of the experiment is shown in Table 1.

Crystalline ergocalciferol (Glaxo Laboratories) with an $E^{1\%}_{1\,cm.}$ of 467 at 265 mμ., was dissolved in 98% ethanol. The appropriate amount of solution was transferred to a palette, the ethanol evaporated off at about 40° and the ergocalciferol taken up in arachis oil and given to the rats.

On each of the 4 succeeding days a group was killed and the pooled livers, intestines and the remainder of the carcasses from each group were taken for assay. The faeces from each group were collected daily. A pooled sample of urine was also collected from a group of rats and assayed as

Table 1. *Design of Exp.* 1

(F = faeces; L = liver; C = 'carcass'; I = intestines.)

Group	No. of rats	Sex	Dose of ergocalciferol (mg.)	Day of collection of faeces: 1st	2nd	3rd	4th	Day of killing	Tissue samples
a	5	4 M., 1 F.	1	F_{a1}	—	—	—	1st	C_a, L_a, I_a
b	5	2 M., 3 F.	1	F_{b1}	F_{b2}	—	—	2nd	C_b, L_b, I_b
c	5	3 M., 2 F.	1	F_{c1}	F_{c2}	F_{c3}	—	3rd	C_c, L_c, I_c
d	5	3 M., 2 F.	1	F_{d1}	F_{d2}	F_{d3}	F_{d4}	4th	C_d, L_d, I_d
e	5	2 M., 3 F.	None	F_{e1}	F_{e2}	F_{e3}	F_{e4}	4th	C_e, L_e, I_e

such, but since no vitamin D was found 24 hr. after dosing no further assays were made.

In the two subsidiary experiments, groups of five rats were also used. In Exp. 2 a group of rachitic rats and a group which received 1 mg. ergocalciferol/rat were killed after 48 hr. and the livers only were assayed. In Exp. 3 two groups of rats were dosed and killed 24 hr. later and the livers from each group, and the faeces from one, were assayed.

Preparation of material

After most of the fur had been shaved off, the rats were skinned and the liver and intestines removed. The latter were split open and the contents removed by rinsing in warm saline. The term 'intestines', therefore, refers to the intestinal tissue only. The contents were discarded to obviate estimating them twice, namely as gut contents and later as faeces. The remainder of the carcasses, including the skin (referred to below as 'carcass') were minced, the individual tissues from each group were pooled and, in Exp. 1, were freeze-dried and kept at $-20°$ until required for assay. In the other two experiments the fresh substances were tested immediately. In all experiments the assays were made on the tissues and faeces as such and not on the unsaponifiable matter.

Method of assay

Vitamin D was estimated biologically on rats by the method of Bourdillon, Bruce, Fischmann & Webster (1931) in which the degree of healing produced is assessed by means of X-ray pictures of the tibiae. In each assay three levels of the International Standard were used and in most instances three levels of the test preparation, six rats being assigned to each dose level. In all about 700 rats were used.

In Exp. 1 five series of assays were carried out between 7 July 1950 and 8 March 1951. The amount of the test preparations administered depended on their expected potency, which had been ascertained approximately in pilot experiments. The following range of dose levels, in mg., of dried material was used: 'carcass', 25–400; liver, 5–100; intestinal tissue, 2·5–200; faeces, 0·5–100; urine, 0·1 ml. The dried preparations from the undosed rats were administered at levels of 500–1000 mg. In assays on fresh material (Exps. 2 and 3) the doses given were approximately equivalent, allowing for water content, to those used for the dried substances.

In assaying the test preparations which had been freeze-dried, appropriate amounts, finely ground, were given to the rats on palettes as a single dose on the first day of the curative period, as also was the standard. When the required dose was less than 40 mg. the samples were diluted with lactose before being weighed out. In every instance these were mixed with a few drops of arachis oil before being administered, as this made the materials more palatable and also prevented loss by scattering. Lactose and oil were also given to all rats which received undiluted doses and to the undosed controls. In Exps. 2 and 3, on fresh material, the liver was weighed out on rice paper and then given to the rats on palettes as a single dose.

RESULTS

The effect of the administration of the massive dose on the development of calcification in the rachitic metaphysis was observed by X-ray examination of the tibiae at daily intervals. No sign of healing was noted on the first day; on the second day a slight trace was visible and by the fourth, healing was appreciable.

Table 2. *Vitamin D assays of tissues and faeces of deficient rats*

Material	Wt. of material: Fresh (g.)	Wt. of material: Dried (g.)	Vitamin D: (i.u./g. dry wt.)	Vitamin D: Total (i.u.)
'Carcass', C_e	51·1	15·2	0·7	10
Liver, L_e	4·30	1·17	1·2	1
Intestines, I_e	4·94	0·77	1·5	1
Faeces, F_{e1}	1·12	0·98	5·0	5
Liver, Exp. 2	3·07	—	0	0

The assays of tissues and faeces of deficient rats (Exps. 1 and 2) showed that virtually no vitamin was present (Table 2). It was therefore assumed

Table 3. *Average weight per rat of tissues and faeces of rats given* 1 *mg. ergocalciferol* (40000 *i.u.*). *Exp.* 1

TISSUES

	1st day (group *a*)		2nd day (group *b*)		3rd day (group *c*)		4th day (group *d*)	
	Fresh (g.)	Dried (g.)	Fresh (g.)	Dried (g.)	Fresh (g.)	Dried (g.)	Fresh (g.)	Dried (g.)
'Carcass'	51·2	15·6	47·2	14·1	55·0	16·8	54·8	16·2
Liver	3·74	1·03	4·16	1·15	4·42	1·22	4·40	1·19
Intestines	4·66	0·69	4·70	0·66	5·30	0·80	5·20	0·76

FAECES

	1st day		2nd day		3rd day		4th day	
Group	Fresh (g.)	Dried (g.)	Fresh (g.)	Dried (g.)	Fresh (g.)	Dried (g.)	Fresh (g.)	Dried (g.)
a	1·24	0·98	—	—	—	—	—	—
b	1·12	0·96	1·08	0·89	—	—	—	—
c	1·06	0·92	0·90	0·81	1·26	1·04	—	—
d	1·08	0·89	0·80	0·70	1·50	1·15	1·62	1·18
Av.	**1·13**	**0·94**	**0·93**	**0·80**	**1·38**	**1·10**	**1·62**	**1·18**

Table 4.* *Vitamin D content per g. dry weight and per whole tissue of rats given* 1 *mg. ergocalciferol* (40 000 *i.u.*). *Exp.* 1

TISSUES

	1st day (group *a*)			2nd day (group *b*)			3rd day (group *c*)			4th day (group *d*)		
Tissue	(i.u./g.)	Total (i.u./rat)	95% fiducial limits (%)	(i.u./g.)	Total (i.u./rat)	95% fiducial limits (%)	(i.u./g.)	Total (i.u./rat)	95% fiducial limits (%)	(i.u./g.)	Total (i.u./rat)	95% fiducial limits (%)
'Carcass'	44·5	690	67–150	44·9†	630	75–133	45·7†	770	71–141	31·1	500	65–154
Liver	1402†	1440	69–144	443	510	67–150	337	410	57–176	339	400	66–151
Intestines	366	250	69–145	142	90	68–147	101	80	54–185	54·1	40	65–154
Total	—	2380	—	—	1230	—	—	1260	—	—	940	—

FAECES

	1st day			2nd day			3rd day			4th day		
Group	(i.u./g.)	Total (i.u./rat)	95% fiducial limits (%)	(i.u./g.)	Total (i.u./rat)	95% fiducial limits (%)	(i.u./g.)	Total (i.u./rat)	95% fiducial limits (%)	(i.u./g.)	Total (i.u./rat)	95% fiducial limits (%)
a	6587	6460	67–150	—	—	—	—	—	—	—	—	—
b	3195	3070	62–161	2249	2000	67–149	—	—	—	—	—	—
c	3987	3670	60–167	1390	1130	57–175	241	250	56–180	—	—	—
d	6992	6220	66–151	1462	1020	66–151	126	150	55–181	21·8	30	56–179
Av.	**5190**	**4855**	—	**1700**	**1383**	—	**184**	**200**	—	**21·8**	**30**	—

* In all tables the potency per g., when less than 100 i.u., was calculated to the first decimal, otherwise to the nearest whole number. The total content in i.u. was rounded off to the nearest 10.

† Weighted geometric mean from replicate assays (see Table 7).

that the amount found in the material obtained from the rats given ergocalciferol was derived from the dose.

Experiment 1

The average weight, per rat, of tissues and faeces before and after freeze-drying is shown in Table 3. Not all the moisture was removed; about 5% remained. The average weight of the rats when killed was about 70 g. Table 4 shows the average vitamin D content per g. of dried tissues and faeces, and also the total content, per rat, of individual tissues and the total faecal excretion, per rat, per day.

The results for the first day after dosing show that, of the 40000 i.u. administered, a total of only about 7000 i.u. could be accounted for. Only one-third of this amount, namely 2380 i.u. was found in the body. A large proportion was present in the liver, the amount being about twice as much as in the remaining 'carcass' and over five times as much as in the intestinal tissue. The faeces from the different groups varied considerably in their vitamin D content, from 3000 to 6000 i.u. The average vitamin content of the faeces was about twice that of the body. No vitamin D was found in urine.

On the second day, the total amount of the vitamin in the tissues and particularly in the faeces had decreased considerably. The fall in the body vitamin was accounted for mainly by a significant decrease of the vitamin in the liver, to about one-third of its value on the first day. The vitamin D in the intestinal tissue had also decreased significantly from 250 to 90 i.u. The vitamin content of the 'carcass' showed practically no change. Though, as on the first day, considerable variation between groups was observed, there was a significant fall in faecal excretion from about 4900 i.u. on the first, to about 1400 i.u. on the second day, a figure approaching the total present in the body.

On the third and fourth days the total vitamin in the tissues did not change significantly from the level observed on the second day. The only significant decrease observed was in the vitamin in the intestine. The faecal excretion of vitamin D was also greatly reduced, to 200 and 30 i.u. respectively.

Considering next the concentrations of the vitamin, i.e. i.u. of vitamin D/g. of fresh weight, it will be noted (Table 5) that on the first day the faeces contained, per g., about 100 times as much as the body, and on the second day about 70 times as much. On the fourth day the concentrations were practically equal, though the total content of the body was 940 i.u. as compared with 30 i.u. in the faeces, in consequence of the greater weight of the former.

The concentration in the individual tissues followed a pattern similar to that of the total content (compare Figs. 1 and 2). Throughout the period, the

Table 5. *Vitamin D concentration in body and faeces of rats given* 40000 *i.u. of ergocalciferol*

(i.u./g. fresh weight.)

	1st day	2nd day	3rd day	4th day
Body	39·9	21·9	19·5	14·6
Faeces*	4293	1536	149	18·5

* The concentration in faeces has been obtained for each individual group and the figure shown is the arithmetic mean.

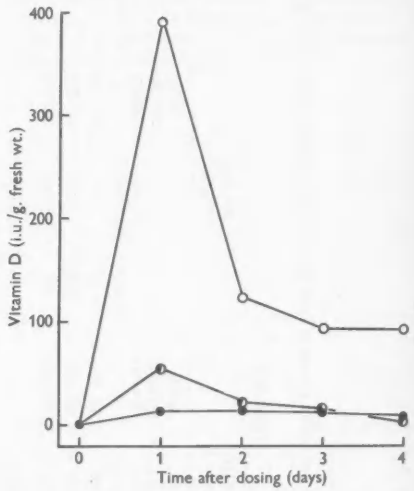

Fig. 1. Vitamin D concentration of fresh tissues of rats given 40 000 i.u. of ergocalciferol. ○—○, liver; ●—●, 'carcass'; ◐—◐, intestines.

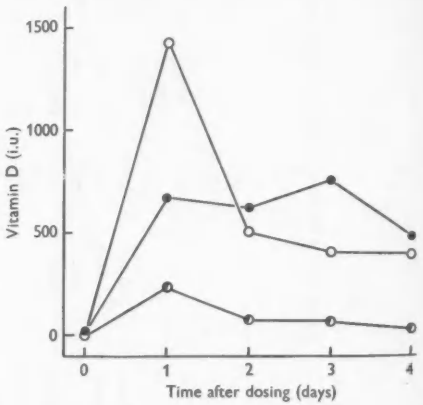

Fig. 2. Vitamin D content of tissues of rats given 40000 i.u. ergocalciferol. ○—○, liver; ●—●, 'carcass'; ◐—◐, intestines.

concentration of the vitamin was much higher in the liver than in the intestines or the 'carcass', particularly on the first day when the liver contained almost 400 i.u./g. fresh weight. The concentration dropped to 120 i.u. on the second day and remained practically at that level. The concentration in the 'carcass' varied very little; it ranged from 9 to 14 i.u./g. of fresh weight. In the intestinal tissue the concentration decreased steadily from 50 i.u. on the first, to 7 i.u. on the fourth day. On the first day it was significantly higher than that of the 'carcass'.

Experiments on fresh material

In the subsidiary Exps. 2 and 3, the assays were carried out on fresh substances. The relevant data and the vitamin D content of the samples tested are shown in Table 6. The total vitamin contents of the fresh livers of rats killed one day after dosing were 1300 and 1680 i.u. respectively. These values are not significantly different from the value of 1440 i.u. found in the main experiment (Table 4) on dried material. Likewise the vitamin D content of the liver on the second day was similar to that of the liver on the corresponding day in Exp. 1. The value obtained for the fresh faeces on the first day fell within the range found in the dried faeces in Exp. 1. The data, therefore, afford no evidence that freeze-drying had materially affected the vitamin D content of either tissues or faeces.

Reproducibility of assays

Replicate assays were made on one specimen of dried liver and on two specimens of 'carcass', the time interval between the original and the replicate assay in each case being 4 months. Reference to Table 7 shows that there is no significant difference between the two estimates of potency on the same sample. There is, therefore, no evidence that loss of vitamin D occurred in the freeze-dried samples during storage at $-20°$ for 4 months.

Recovery of dose administered

Table 8 was compiled from the results of all experiments, that is, on both fresh and dried preparations, and shows the percentage of the dose recovered in the tissues and faeces together with the 95 % fiducial limits. It will be seen that, of the 40000 i.u. administered, a total of 20·4 % was accounted for in the body and faeces on the first day, two-thirds being present in the faeces. On the 3 subsequent days the proportions found in the faeces decreased steadily to 0·1 % on the fourth day. For assessing the total recovery, the excretion on the second day has been included. The excretion on the last 2 days contributed little to the total recovery. A further possibility is that it may not have been derived directly from the dose, but indirectly from re-excretion into the gut through the bile.

DISCUSSION

The reasons for the low recovery of the dose, amounting to only 20–30 %, had to be considered. A loss could have occurred during the preparation of the calciferol for dosing, or through incomplete uptake of the dose by the rats. Losses might also have occurred on freeze-drying, and during storage of the materials. Since great care was taken in the preparation of the ergocalciferol solution and in

Table 6. *Vitamin D content of fresh liver and faeces of rats given* 1 *mg. ergocalciferol* (40000 *i.u.*). *Exps.* 2 *and* 3

Exp. no.	No. of rats	Material	Fresh wt. (g.)	Vitamin D content: 1st day (i.u./g.)	1st day Total (i.u.)	2nd day (i.u./g.)	2nd day Total (i.u.)	95% fiducial limits (%)
2	5	Liver	3·20	—	—	141	450	75–134
3	5	Liver *a*	4·00	324	1300	—	—	71–141
	5	Liver *b*	4·10	411	1680	—	—	71–140
	5	Faeces	1·07	6135	6560	—	—	66–151

Table 7. *Comparison of replicate assays on dried material*

Date of assay	Material	Code no.	Vitamin D content (total i.u.): 1st day	2nd day	3rd day	95% fiducial limits (%)
7. vii. 50	Liver	L_a	1900	—	—	56–179
8. xi. 50	Liver	L_a	1210	—	—	63–160
8. xi. 50	'Carcass'	C_b	—	620	—	67–149
7. iii. 51	'Carcass'	C_b	—	650	—	67–149
10. xi. 50	'Carcass'	C_c	—	—	710	53–190
7. iii. 51	'Carcass'	C_c	—	—	790	67–149

Table 8. *Percentage of dose* (1 *mg. ergocalciferol*) *recovered in tissues and faeces* (*Exps.* 1–3)

Tissue	1st day	2nd day	3rd day	4th day
	Recovery of dose			
	(%)	(%)	(%)	(%)
'Carcass'	1·7	1·6	1·9	1·3
Liver	3·7	1·2	1·0	1·0
Intestines	0·6	0·2	0·2	0·1
Total	6·1	3·0	3·1	2·4
95% fiducial limits	5·1–7·2	2·5–3·6	2·4–4·2	1·8–3·2
Faeces*	14·3	2·7	0·5	0·1
95% fiducial limits	10·6–19·3	2·1–3·4	0·33–0·75	0·05–0·18

* Estimated mean percentage recovery and fiducial limits from the regression line.

ensuring that it was completely consumed by the rats, it is unlikely that a loss of 70–80 % could have occurred during the dosing procedure. Had a loss occurred at this stage, it is likely that erratic results would have been obtained in the different experiments, which was not the case. Losses during freeze-drying and storage could be ruled out as the result of experiments already referred to.

In addition to these control experiments, comparisons were made between the potency of fresh livers assayed as such and as the unsaponifiable matter obtained therefrom. Since concordant results were obtained, the simpler method of assaying the material was adopted.

From the present experiments it appears that the liver, both as regards content and concentration, is one of the main sites of absorbed vitamin, at least during the short period investigated and in the special conditions of our tests. This is in contrast with the results of Morgan & Shimotori (1943), who found that the liver of dogs is capable of only moderate storage, some of the other tissues having a higher concentration. On the other hand, Heymann (1937) states that in rabbits the liver can hold vitamin D more tenaciously than can the other organs.

In our experiments the high initial content of the liver was not maintained; a significant drop occurred on the second day. It is difficult to trace the loss of this 1000 i.u., but it does not appear to have been transferred to the other parts of the body. The possibility exists that it may have been changed into inactive products; alternatively, it may have been re-excreted into the gut via the bile. This, however, seems unlikely, since the bulk of the faecal vitamin D on the second day would then be derived from this re-excretion.

In view of the laborious nature of the assays, it was not possible in this investigation to examine the distribution of the vitamin in the different organs and tissues of which the 'carcass' is comprised. It should be noted, however, that on the first day, one-third of the absorbed vitamin was already present in the 'carcass'. Little change in the content took place throughout the experimental period. The concentration was low, but this does not rule out the possibility that some organs, though small in size, may have had a rather high concentration of the vitamin. Later work (Cruickshank & Kodicek, 1952*b*) has indeed shown that, on the second day after dosing, kidney, adrenals, spleen and lungs contained concentrations of the vitamin similar to that of the liver.

The vitamin D content of the intestinal tissue reflected the amount passing through the alimentary tract, but it was low in proportion to the amount offered. On the first day the intestinal tissue contained about 6 μg. while 1000 μg. had been ingested.

The faeces contained a considerable amount of vitamin D, which decreased steadily throughout the experimental period. Nevertheless, the value on the fourth day showed that excretion had not ceased, since this amount was significantly higher than that found in the faeces of the undosed rats. The vitamin D occurring in the faeces on the last 2 days of the experiment might possibly have been derived from absorbed vitamin D which had been re-excreted into the gut, e.g. in the bile. This view is supported by the experiments of Heymann (1937–8) who stated that ingested vitamin D may appear later in the bile. On the other hand, Taylor, Weld & Sykes (1935) and Morgan & Shimotori (1943) found no evidence of re-excretion in dogs following administration of a massive dose. To ascertain the rate of movement of the ingesta, Sudan III was administered to a group of rachitic rats on the Steenbock diet. It was observed that the dye was still present in the faeces after 2 days. Possibly, therefore, traces of the vitamin derived from the dose were present in the excreta on the third day, though the passage of Sudan III may not necessarily correspond with that of the vitamin.

As previously mentioned, no vitamin D was detected in urine by biological assay. The possibility existed that it had been excreted in a conjugated

form. However, since the ether extract of urine, hydrolysed according to the method of Bachman & Pettit (1941) for conjugates of steroid hormones, showed no biological activity, it is considered unlikely that any appreciable amount of the vitamin had been excreted in this form.

From the data obtained, it appears that the total recovery in body and excreta amounted to only 23 %, or taking the extreme fiducial limits, to 30 %, so that about 70–80 % of the dose could not be accounted for. The fate of this large deficit is at present unknown, but it was presumably converted into inactive products not detectable by biological assay. This destruction or conversion might occur in the alimentary tract, in some body tissue, or in the faeces after excretion. It has been reported that cholesterol can be destroyed by certain micro-organisms (Turfitt, 1944; Wainfan, Henkin, Rice & Marx, 1952; Stadtman & Cherkes, 1952), but it is not known whether vitamin D can be so attacked. If it should be so, the destruction might occur in the caecum or in some other part of the digestive tract or, alternatively, in the faeces, which, in our experiments, after being voided, were exposed to a temperature of 21° in the experimental rat room until collected at 24 hr. intervals.

The results obtained in the particular conditions of the experiment are of special interest in view of the use of massive doses of vitamin D for the prevention and cure of rickets in infants.

SUMMARY

1. A massive dose of ergocalciferol (40000 i.u.) was given *per os* to groups of weanling rachitic rats and on each of the 4 succeeding days the pooled livers, intestines (minus contents), the remainder of the carcasses, the faeces and the urine were assayed biologically for vitamin D. Forty rats were used in the preparation of the material to be tested, and over 600 for the assays.

2. The assays were performed on freeze-dried, and in some instances, on fresh material. There was no evidence that loss of the vitamin occurred on freeze-drying or storage.

3. Little or no vitamin D was detected in rachitic undosed animals. The estimates of the vitamin D content of the body of rats receiving calciferol were 2380, 1230, 1260 and 940 i.u. on the 4 consecutive days, respectively. The decrease on the second day after dosing was mainly due to a fall in the vitamin in the liver, from a value of 1440 to 510 i.u., a level subsequently maintained. The intestinal tissue showed a steady decrease from 250 on the first, to 40 i.u. on the fourth day. The remainder of the carcass maintained a level of about 600 i.u. throughout the experimental period.

4. It appeared that the liver was the main site of deposition. No significant transfer of the vitamin from liver to other tissues of the body was observed within the period studied.

5. The estimated faecal excretion of vitamin D which was 4860 i.u. on the first day decreased to 1380, 200 and 30 i.u. on the subsequent days. No vitamin D was found in urine, assayed as such or as the ether extract of urine hydrolysed with concentrated hydrochloric acid.

6. About 23 % of the dose was accounted for. The fate of the remainder of the ingested vitamin is not known. It is assumed that it is converted into products having no antirachitic activity.

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PART II. STATISTICAL ANALYSIS

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This second part describes the statistical analysis of the experimental results presented in the preceding section, which gives a general account of the work.

Vitamin D assays

Exp. 1. Of the twenty-nine preparations shown in Table 1 of the preceding article (Cruickshank & Kodicek, 1953) all except F_{e2}, F_{e3} and F_{e4} were assayed against a standard preparation of ergocalciferol. The assays were carried out on five occasions, denoted here by assays I–V. The allocation of the test preparations to the five assays is shown in Table 1. On each occasion the standard preparation was used at three dose levels (3·5, 7 and 14 i.u.). Three preparations (L_a, C_b and C_c) were assayed twice.

An analysis of variance of the responses (X-ray scale readings) was performed for each assay separately. A typical analysis (that for assay I) is shown in Table 2. The main features of each assay—the common slope of the parallel response-log dose regression lines, the residual mean square, and the variance ratios corresponding to non-parallelism and non-linearity—are shown in Table 3. There are significant differences between the five assays, both in the slopes and in the residual mean squares; no attempt, therefore, was made to analyse the data collectively by using a pooled value for the slope or for the residual variance. Four of the five variance ratios for non-parallelism shown in Table 3 are higher than expectation, but none reaches significance at the 0·05 level, and further examination shows that the differences between slopes, within days, are collectively not significant.

Table 1. *Allocation of test and standard preparations in vitamin D assays* (*Exp.* 1)

Assay	Test preparations	Standard
I	C_a, L_a, I_a, F_{a1}, F_{d1}, F_{d2}	Ergocalciferol
II	C_b, L_a (repeat), L_b, I_b, F_{b2}	
III	C_c, L_c, I_c, F_{c2}, F_{d3}	
IV	C_b (repeat), C_e (repeat), C_d, C_e, I_d, I_e, L_e	
V	L_d, F_{b1}, F_{c1}, F_{e1}, F_{e3}, F_{e4}	

For notation, see Table 1 of preceding article.

Table 2. *Analysis of variance for assay I*

	Sum of squares	Degrees of freedom	Mean square	Variance ratio
Between preparations	242·3428	6	40·3905	44·3 ($P<0·001$)
Within preparations				
Due to common slope	130·3001	1	130·3001	142·7 ($P<0·001$)
Between slopes	11·4263	6	1·9044	2·09 ($0·5<P<0·10$)
Deviations from linearity in individual preparations	5·9525	9	0·6614	<1 ($P>0·20$)
Within doses	114·9892	126	0·9126	—
Total	505·0109	148		

Table 3. *Summary of vitamin D assays for Exps.* 1–3

Exp.	Assay	Common slope ±standard error	Residual mean square (with degrees of freedom)	Variance ratios, and significance: Between slopes	Variance ratios, and significance: Departures from linearity
1	I	3·19±0·27	0·9126 (126)	2·09 ($0·05<P<0·10$)	<1 ($P>0·20$)
	II	4·73±0·50	1·4269 (84)	2·17 ($0·05<P<0·10$)	<1 ($P>0·20$)
	III	3·04±0·50	1·0155 (73)	1·14 ($P>0·20$)	<1 ($P>0·20$)
	IV	4·28±0·50	1·1406 (83)	1·84 ($0·10<P<0·20$)	<1 ($P>0·20$)
	V	4·36±0·67	1·2337 (68)	<1 ($P>0·20$)	<1 ($P>0·20$)
2	VI	6·96±0·69	1·7472 (30)	<1 ($P>0·20$)	<1 ($P>0·20$)
3	VII	5·27±0·46	1·5598 (65)	<1 ($P>0·20$)	<1 ($P>0·20$)

The potency of each preparation was calculated in the usual way, and the total vitamin content of each tissue or specimen of faeces was obtained by multiplying by the dried weight. The results are shown in Tables 2 and 4 of the preceding article. The approximate 95% fiducial limits shown in Table 4 were obtained in the usual way from the variance of the log potency ratio (Emmens, 1948). The true potency may be expected to lie between the two limits about 19 times out of 20.

For each of three preparations (L_a, C_b and C_c), two estimates of potency were available (Table 7 of the preceding article). In no instance did any pair of estimates differ significantly, and the combined estimates shown in Table 4 of the preceding article were obtained by weighting the separate estimates of log potency in inverse proportion to their variances, calculating a weighted mean, and taking its antilogarithm. This procedure is equivalent to calculating a weighted geometric mean of the separate potency estimates. The variance of the combined log potency ratio was estimated as the reciprocal of the sum of the weights, and approximate fiducial limits calculated as before.

Exps. 2 *and* 3. The single test preparation of Exp. 2 (liver, 48 hr. after ingestion) was assayed against a standard (assay VI), as were the three test preparations of Exp. 3 (liver from two groups and faeces from one, 24 hr. after ingestion) (assay VII). The slope, residual variance and variance ratios for non-parallelism and non-linearity are shown for each assay in Table 3. The potencies and fiducial limits were calculated as in Exp. 1, and are shown in Table 6 of the preceding article.

Distribution of vitamin D in the tissues

The significance of differences between the vitamin D content of, say, the 'carcass' on the first 4 days after ingestion cannot, unfortunately, be determined purely from the sampling error of the potency estimates (as given in Table 4 of the preceding article): the four 'carcass' preparations came from different groups of rats, and there will inevitably be a certain amount of biological variation between these four groups. For example, the vitamin content of the liver shows a significant fall between the first day (1440 i.u.) and the second day (510 i.u.). This result does not necessarily mean that a single large group of rats would show a fall in the average vitamin content of the liver between the first and second days after ingestion: it may reflect merely a difference in behaviour between the rats of group *a* (which were killed on the first day) and those of group *b* (killed on the second day), such as might have occurred between any two groups selected at random had they been killed on the same day. The same difficulty does not apply to the faeces determinations, for on each day (except the fourth) there are results from more than one group of rats, and the biological variation between the groups can be assessed.

Liver content. The difficulty mentioned above, of interpreting the fall in liver content between the first and second days after ingestion, was the reason for carrying out Exps. 2 and 3. Exps. 1–3 together provide estimates of liver content for three groups killed on the first day after ingestion, and for two groups killed on the second day. Variations between groups may therefore be taken into account in the comparison of the first and second days.

The vitamin contents shown in Tables 4 and 6 of the preceding article are expressed in Table 4 as percentages of the total dose of 40 000 i.u. The result of a *t* test applied to the logarithms of these percentages is shown in Table 4: the difference between the 2 days is highly significant ($P < 0{\cdot}01$). The means of the logarithms of percentage recovery are 0·5639 (assay I) and 0·0790 (assay II). Taking the antilogarithms of these figures (which is equivalent to calculating the geometric mean of the individual values of percentage recovery), we obtain the values 3·7 and 1·2%, which are given in Table 8 of the preceding article.

'Carcass' content. The vitamin content of the 'carcass' on each of the 4 days is given in Table 4 of the preceding article, and is also expressed in Table 8 as a percentage of the initial dose of 40 000 i.u. The significance of changes in the percentage recovery over the 4 days was determined by a regression analysis of log (percentage recovery) on days. Each of the four values of log (percentage recovery) was weighted in inverse proportion to its sampling variance (which is the same as that of the corresponding log potency ratio). The result of the analysis is shown in Table 5. On the hypothesis that apparent differences between days are due only to sampling fluctuations of the animals used in the

Table 4. *Test for difference between vitamin D contents of liver on first and second days after ingestion*

Assay I			Assay II		
Exp.	% recovery	log	Exp.	% recovery	log
1 (L_a)	3·610	0·5574	1 (L_b)	1·273	0·1048
3 (*a*)	3·237	0·5101	2	1·130	0·0531
(*b*)	4·208	0·6241			
Total		1·6916	Total		0·1579
	Mean log = **0·5639**			Mean log = **0·0790**	

Difference in means = 0·5639 − 0·0790 = 0·4849.
S.E. (difference) = 0·0468.
$t = 0{\cdot}4849/0{\cdot}0468 = 10{\cdot}35$ on 3 D.F. ($P < 0{\cdot}01$).

Table 5. *Analysis of regression of log (percentage recovery) in 'carcass', on days after ingestion*

	Sum of squares (χ^2)	Degrees of freedom	Mean square	Variance ratio
Due to regression	0·3974	1	0·3974	<1 ($P > 0{\cdot}20$)
About regression	2·0801	2	1·0401	—
	2·4775	3		

Table 6. *Analysis of regression of log (percentage recovery) in intestines, on days after ingestion*

	Sum of squares (χ^2)	Degrees of freedom	Mean square	Variance ratio
Due to regression	39·0826*	1	39·0826	27·70 ($0·01 < P < 0·05$)
About regression	2·8219	2	1·4110	—
	41·9045*	3		

* χ^2 highly significant ($P < 0·001$).

Table 7. *Analysis of regression of log (percentage recovery) in faeces, on days after ingestion*

	Sum of squares (χ^2)	Degrees of freedom	Mean square	Variance ratio
Due to regression	443·5499*	1	443·5499	176·23 ($P < 0·001$)
About regression	6·4619†	2	3·2309	1·28 ($P > 0·20$)
Between replicates on same day	17·6184†	7	2·5169	—
	467·6302	10		

* χ^2 highly significant ($P < 0·001$). † χ^2 significant ($0·01 < P < 0·05$).

assay, the sums of squares would be distributed as χ^2, with degrees of freedom as shown in Table 5. The overall sum of squares, and also the components due to regression and about regression, are non-significant. The differences between days may therefore safely be ascribed to sampling fluctuations.

Intestinal content. The vitamin content of the intestines on each day is shown in Table 4 of the preceding article, and also, as a percentage recovery of the initial dose, in Table 8. A weighted regression analysis, similar to that for 'carcass', was carried out, and the results are shown in Table 6. The sum of squares due to regression, regarded as χ^2 on one degree of freedom, is highly significant ($P < 0·001$). Moreover, the variance ratio between the mean square due to regression and that about regression is significant ($0·01 < P < 0·05$); the significance of the regression component is therefore not due merely to differences between groups of experimental animals. We can safely assert the existence of a fall in intestinal vitamin content with successive days after ingestion.

Faeces content. Tables 4 and 6 of the preceding article provide in all five observations of the vitamin content of the faeces on the first day after ingestion (four from Exp. 1, and one from Exp. 3), three observations for the second day, two for the third day, and one for the fourth day. A weighted regression analysis of log (percentage recovery) on days was performed. This analysis, unlike those for the 'carcass' and intestinal contents, provides a direct estimate of the variation between experimental groups. (The fact that some experimental groups provided observations on more than one day after ingestion has been ignored in the analysis: it appears by inspection to be an unimportant factor.)

The analysis of variance is shown in Table 7. The total sum of squares, and each of its components, are significant when judged as χ^2 values. The significance of the sum of squares between replicates indicates biological variation between the groups: the apparent differences in vitamin content between groups examined on the same day after ingestion cannot be explained purely by assay sampling error. The significance of the differences between the 4 days must therefore be judged by comparison with the mean square between replicates. The variance ratio about regression is not significant, showing that deviations from a linear regression line could easily arise by chance in view of the variation between replicates. The variance ratio for regression is highly significant. We conclude, therefore, that the vitamin content of faeces tends to fall with successive days after ingestion, and may well do so by a constant proportion on each day.

The mean values of log (percentage recovery) on each day were estimated from the regression line. The corresponding antilogarithms are shown in Table 8 of the preceding article, together with their 95% fiducial limits. These limits should include, with a probability of 0·95, the true geometric mean percentage recovery of a large number of experimental groups examined on each day. It may be observed that on each day the percentage recovery falls to about 20% of its value on the previous day.

Total recovery of dose. The estimates, shown in Table 8 of the preceding article, of total percentage recovery in the tissues are obtained simply by adding the separate estimates for the three tissues. Fiducial limits for such estimates, which take into account variation between experimental groups, could be obtained, as with faeces, by fitting a regression line, and using the estimate of residual variation about the line. As, however, we have only one observation on each day, such a procedure would lead to very wide limits. It therefore seemed more useful to give in Table 8 the 95% fiducial limits based only on the assay variation. These limits include, with a probability of 0·95, not the mean recovery over a large number of groups, but the actual recovery in the particular groups used in this experiment—which could have been determined to any degree of accuracy by increasing the number of animals used in the assays.

Let y_C, y_L and y_I denote, respectively, the estimated percentage recovery in 'carcass', liver and intestines, on any one day. The estimated total recovery in the tissues is $y = y_C + y_L + y_I$. Denote by V_C, V_L and V_I the variances of the logarithms of y_C, y_L and y_I respectively (these are equal to the variances of the log potency-ratios found in the assays). Then the variance of $\log y$, denoted by V, is given approximately by

$$V = \frac{y_C^2 V_C + y_L^2 V_L + y_I^2 V_I}{y^2}.$$

95% fiducial limits for y are given by the antilogarithms of $\log y \pm 1·96\sqrt{V}$. The limits shown in Table 8 were calculated by this method.

We wish to thank Mr A. L. Bacharach, Glaxo Laboratories Ltd., for the gift of crystalline ergocalciferol, and the Superintendent of the Low Temperature Station for the facilities provided for drying and storage of material. We are indebted to Mr S. G. Impey, Miss N. Gass and Mr D. R. Ashby for their valuable technical assistance, and to Miss I. K. Allen for computing assistance.

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Pancreatic Trypsin Inhibitor

2. REACTION WITH TRYPSIN

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The reactions of trypsin with its inhibitors are among the few known cases of specific interaction between two purified proteins (Kunitz & Northrop, 1936; Kunitz, 1947; Fraenkel-Conrat, Bean & Lineweaver, 1949; Fraenkel-Conrat, Bean, Ducay & Olcott, 1952; Schmitz, 1939; Laskowski & Laskowski, 1951). Under favourable conditions, the inhibitor-trypsin compound is formed extremely easily; thus, we have observed that when aqueous suspensions of pancreatic inhibitor crystals and trypsin crystals were allowed to merge on a microscope slide under a cover-slip, the characteristic crystals of inhibitor-trypsin compound formed rapidly in the boundary zone. The course of the reaction in solution can be studied, since the resulting compound is devoid of tryptic activity. This study, besides yielding information on the mode of reaction of trypsin with inhibitor and possibly with substrate, might also yield information on the interaction of proteins in general.

Kunitz & Northrop (1936) found that the pancreatic inhibitor (mol.wt. 6000) combined at pH 7 with about an equimolecular amount of trypsin (mol.wt. 34 000); the resulting compound was completely dissociated at pH 1 and reformed at higher pH. Both combination and dissociation required 'measurable time intervals', but kinetic data were not published. Grob (1949), using 10^{-9} M solutions, found the dissociation constant of the compound to be approximately 6×10^{-10} M.

In the present paper, detailed results are given of an investigation on the nature and course of reaction between trypsin and the crystalline pancreatic inhibitor whose properties and composition were described by Green & Work (1953). A method for following the course of reaction is described and the effect of pH on reaction velocity considered in particular.

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EXPERIMENTAL AND RESULTS

Trypsin, inhibitor and haemoglobin substrate were all crystalline preparations obtained as described by Green & Work (1953). The method of estimation of trypsin activity (adapted from Anson, 1938) was also as described, the degree of digestion of denatured crystalline carboxyhaemoglobin over 5 min. at pH 7·5 and 25° being measured by the optical density at 280 mμ. of the deproteinized solution. Unless otherwise stated, final trypsin concentration in the reaction mixture was 19 μg./ml. (1 trypsin unit (T.U.)/ml.) Measurement of protein concentration and definition of trypsin and inhibitor units (I.U.) were also as described. Since combination of inhibitor with trypsin under the conditions of experiment was stoicheiometric and almost irreversible, the trypsin activity of a mixture of the two compounds represented a measure of the uncombined trypsin.

Dissociation constant

This was calculated by investigating in detail near the equivalence point an inhibition curve (Fig. 1) obtained by adding increasing amounts of inhibitor to a fixed amount of trypsin at a greater dilution (4·75 μg./ml.) than that normally employed, so that dissociation would be more marked. The residual trypsin activity (0·2 unit) in the presence of one equivalent of inhibitor was used to calculate an approximate dissociation constant for the equilibrium, $IT \rightleftharpoons I + T$ (in which I = inhibitor and T = trypsin).

Table 1. *Effect of variation of haemoglobin concentration on inhibitor activity*

(Trypsin concentration 9·2 μg./ml. All haemoglobin solutions contained 32% (w/v) of urea.)

Haemoglobin concentration* (%, w/v)	0·41		0·82		1·65	
Inhibitor added (ml.)	0·15	0·3	0·15	0·3	0·15	0·3
Inhibition (%)	24	65	22	69	22	64

* Concentration of haemoglobin required to saturate trypsin = 1·1%.

Assuming the value 34000 for mol.wt. of trypsin,

$$1 \text{ T.U./ml.} = 5{\cdot}5 \times 10^{-7} \text{ mole/l.};$$

from Fig. 1,

$$[I] = [T] = \frac{0{\cdot}02}{2} \text{ T.U./ml.}$$

(total vol. of digest = 2 ml.)

$$[IT] = \frac{0{\cdot}51 - 0{\cdot}02}{2} = 0{\cdot}245 \text{ T.U./ml.}$$

therefore

$$K = 5{\cdot}5 \times 10^{-7} \times \frac{0{\cdot}01^2}{0{\cdot}245} = 2{\cdot}2 \times 10^{-10} \text{M}.$$

A second experiment gave $K = 3 \times 10^{-10}$ M.

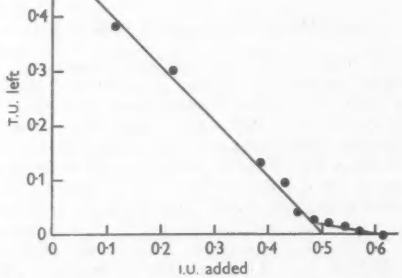

Fig. 1. Inhibition curve, illustrating the dissociation at pH 7·5 of the inhibitor-trypsin compound near the equivalence point. Trypsin concentration in final reaction mixture 4·75 μg./ml.

Variation of substrate concentration

In an attempt to decide whether inhibition of trypsin by the pancreatic inhibitor was competitive or non-competitive, determinations were made of inhibition by two different amounts of inhibitor in the presence of three different substrate concentrations. The concentrations of substrate and urea were maintained constant, and calibration curves of trypsin activity were plotted for each haemoglobin concentration. Table 1 shows that inhibition was independent of substrate concentration over a four-fold variation of concentration. When the haemoglobin concentration was further raised, the results were rendered inconclusive because of partial precipitation of the haemoblobin during the digestion.

Velocity constant

In the usual determination of inhibitor activity by constructing inhibition curves (Green & Work, 1953), inhibitor and trypsin were allowed to react for 5 min. before the addition of substrate. The resulting digestion value was then proportional to the

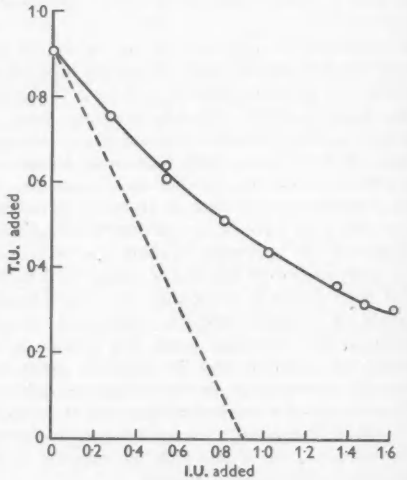

Fig. 2. Effect of order of addition of reactants on inhibition curve. ○—○, Trypsin added to mixture of haemoglobin and inhibitor; – – –, standard type of curve which would have resulted if haemoglobin had been added to a mixture of trypsin and inhibitor after 5 min. standing at 25°.

difference between trypsin and inhibitor concentrations ($[T] - [I]$). If, on the other hand, trypsin was added to a mixture of inhibitor and substrate, it was found that the observed inhibition was much lower (Fig. 2). This is readily understood if the reaction between inhibitor and trypsin is a slow one, as indicated by Kunitz & Northrop (1936). The trypsin activity at the beginning of the 5 min. digestion period would then be that of the initial free trypsin, while the activity at the end would be

approximately $[T]-[I]$. The observed digestion value would be a measure of the average trypsin activity during 5 min.; moreover, it would be some function of the initial concentrations of free inhibitor and free trypsin, and hence could be used to determine the extent of reaction in a partially reacted mixture of inhibitor and trypsin. In order to follow the reaction between inhibitor and trypsin, a calibration curve was determined (Fig. 3), showing the degree of digestion of haemoglobin in the presence of known equivalent amounts of trypsin and inhibitor. Digestion flasks were prepared containing substrate and different amounts of inhibitor; trypsin, equivalent in amount to the inhibitor present, was added to each flask and the degree of digestion after 5 min. determined in the usual way.

A velocity constant for the reaction between inhibitor and trypsin in the presence of haemoglobin was calculated from the calibration curve. In view of the low dissociation constant of the trypsin-inhibitor complex, the velocity of dissociation of this complex was neglected.

Let a = initial inhibitor concentration
= initial trypsin concentration.
$a-x$ = trypsin activity left after time t.
k = 2nd order velocity constant (ml./T.U./min.).

$$-\frac{dx}{dt}=k(a-x)^2,$$

therefore
$$kt=\frac{1}{a}\frac{x}{a-x}, \qquad (1)$$

$$x=\frac{a^2kt}{1+akt}.$$

Average trypsin activity during t min.

$$=\frac{1}{t}\int_0^t (a-x)\,dt,$$

substituting for x

$$=\frac{a}{t}\int_0^t \frac{1}{1+akt}\,dt$$

$$=\frac{1}{kt}\ln(1+akt). \qquad (2)$$

The dotted line in Fig. 3 was obtained by plotting equation (2) using a value for k of 1·05 ml./T.U./min. ($3{\cdot}1\times10^4$ l./mole/sec.). (k is expressed in two types of units, owing to uncertainty over the mol.wt. of trypsin.)

As there was reasonable agreement between the calculated curve and the observed points, this value of k was used to calculate the time of half reaction ($1/kt$) and gave a value of about 2 min. In contrast, preliminary experiments showed that the inhibitor-trypsin reaction in the presence of buffer at pH 7 was almost complete in 1 min. There was thus a considerable discrepancy between the time of the inhibitor-trypsin reaction in the presence and in the absence of substrate.

Effect of urea. The explanation of this discrepancy could not lie in the effect of substrate on the reaction, since haemoglobin concentration had already been shown to have no effect on inhibition (Table 1). There was the possibility that the high

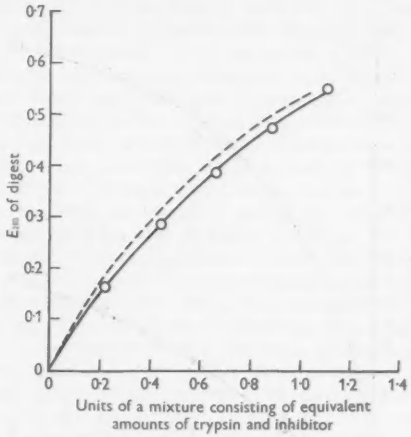

Fig. 3. Calibration curve for determining the amount of free inhibitor present in a partially reacted trypsin-inhibitor mixture. O—O, digestion values obtained from the reaction of equivalent amounts of trypsin and inhibitor for 5 min. in the presence of 1 ml. of substrate solution; – – –, plot of equation (2) using $k=1{\cdot}05$ ml./T.U./min.

urea content of the substrate solution might be affecting reaction velocity. Determination of the effect of 14 % urea on the rate of reaction of trypsin with inhibitor in buffer at pH 7 showed that this was the correct explanation; inhibitor was mixed with buffered trypsin (pH 7) in the presence or absence of urea; at 10 sec. intervals after mixing, 1 ml. portions of these mixtures were added to 1 ml. of substrate solutions so adjusted as to give identical final urea concentrations in the two digests. The digestion value after 5 min. was measured as usual, and the degree of reaction for each point read from the calibration curve (Fig. 3). The velocity constants k for the two reaction mixtures were determined by means of equation (1) from the slope (ka) of the lines obtained by plotting t against $x/(a-x)$ (Fig. 4). The value 0·86 ml./T.U./min. ($2{\cdot}6\times10^4$ l./mole/min.) obtained in the presence of urea was of the same order as that determined by calculation from Fig. 3. In the absence of urea k was 6·9 ml./T.U./min. (21×10^4 l./mole/min.), confirming that urea lowered the velocity of the inhibitor-trypsin reaction.

Effect of acid reaction. To test the effect of change in pH in the acid region on the velocity of the inhibitor-trypsin reaction, the time course of reactions at pH 3, 4, 5, 5·9 and 7 were determined by mixing equivalent amounts of trypsin and inhibitor in 0·02 M-citrate-phosphate buffer of the required pH, testing trypsin activity of portions at various time intervals and calculating percentage reaction from Fig. 3. The results (Fig. 5) show that as the pH

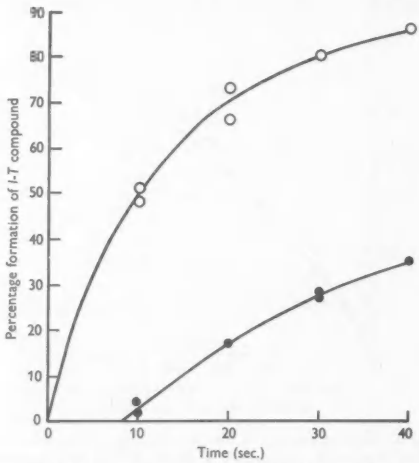

Fig. 4. Effect of urea on rate of reaction between inhibitor and trypsin at pH 7·0. 0·5 ml. trypsin (6 T.U./ml.) added to 2·4 ml. 0·01 M-phosphate buffer pH 7 + 0·55 ml. inhibitor (5·6 I.U./ml.) + 1·55 ml. of either urea (45%, w/v) or water. 1 ml. samples of these reaction mixtures were added at 10 sec. intervals to 1·0 ml. of substrate solution containing 1·2% haemoglobin and either 16 or 30% urea, the final urea concentrations being the same in each case. Resultant degree of reaction obtained from digestion value using calibration curve (Fig. 3). ●—●, reaction in presence of 14% urea; ○—○, normal reaction without urea.

was lowered the velocity dropped rapidly between pH 7 and 5·9; there was little change between pH 5·9 and 5, but at pH 4 the velocity was further reduced. At pH 3 there was no measurable reaction, the resulting digestion values being identical with the control values obtained by adding the same amount of trypsin to a mixture of inhibitor and substrate.

Effect of alkaline reaction. This was less easy to investigate than acid reactions owing to the ready alkaline denaturation of trypsin. It was first necessary to determine if denatured trypsin combined with inhibitor; accordingly, inhibition curves were constructed with trypsin which had been partially denatured by either heat or alkali; Fig. 6 shows the slope of these lines to be the same (within 10%) as that with control untreated trypsin; therefore, denatured trypsin had a negligible effect on the inhibitor-trypsin combination.

The effect of alkaline reaction on the combination was estimated by allowing trypsin to react with different amounts of inhibitor in glycine-sodium hydroxide buffers at pH 12·4, 11·8, 10·5 for 1 min.

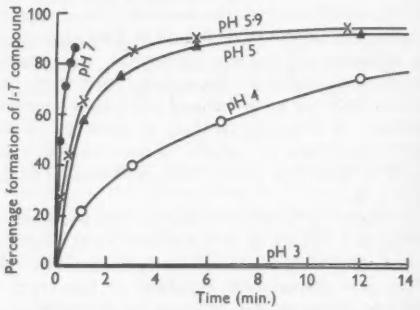

Fig. 5. Effect of acid pH on velocity of inhibitor-trypsin reaction. Equivalent amounts of trypsin and inhibitor reacted in 0·02 M-citrate-phosphate buffer; trypsin activity tested on portions after times shown. Degree of reaction calculated from Fig. 3. Trypsin concentration in inhibitor-trypsin reaction mixture 43 μg./ml., except in case of pH 7 reaction where it was 22 μg./ml.

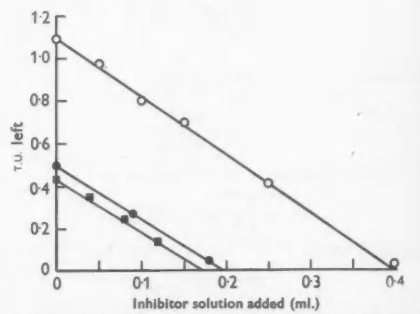

Fig. 6. Inhibition curves showing effect of partial denaturation of trypsin on its combination with inhibitor. ○—○, native trypsin; ●—●, trypsin heated at 50° at pH 7 for 5 min.; ■—■, trypsin exposed to pH 12 for 1 min.

before addition of substrate. With the more alkaline buffers, it was necessary to add a predetermined amount of acid to the substrate to bring the pH of the final digestion mixture to 7·5. Owing to alkaline inactivation of trypsin, no determinations of velocity could be made; the results presented in Fig. 7 show inhibition curves for the various reactions moved vertically so that they start from the same ordinate to facilitate comparison. The circles on the upper and lower curves are control points

showing respectively the effect of no combination (trypsin added to haemoglobin and inhibitor) and of almost complete combination at pH 7. In order to relate the results to those obtained at acid reaction, a similar inhibition curve was plotted at pH 4; this curve and that obtained at pH 12·4 were almost identical with the control 'no reaction' curve showing that there was very little reaction at

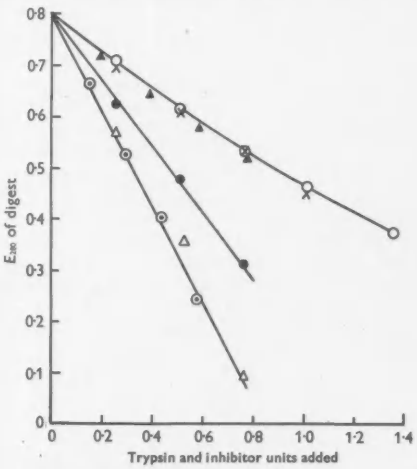

Fig. 7. Effect of alkaline pH on inhibitor-trypsin reaction. Scales adjusted to give common origin. Trypsin (15 μg./ml.) reacted with equivalent amounts of inhibitor in glycine-NaOH buffer for 1 min. before addition of substrate at pH 7·5. Usual 5 min. digestion period. ○, no reaction, control, trypsin added to inhibitor and haemoglobin; ×, pH 4; ▲, pH 12·4; ●, pH 11·8; △, pH 10·5; ⊙, pH 7, complete reaction.

pH 12·4 in 1 min. The curve obtained at pH 10·5 was almost identical with that at pH 7, while at pH 11·8 the degree of reaction was intermediate between the two controls.

DISCUSSION

The value of 2×10^{-10} M for the dissociation constant at pH 7 of the inhibitor-trypsin compound is probably correct only within an order of ten on account of the very small measured value from which it was calculated (residual trypsin activity in the presence of one equivalent of inhibitor, Fig. 1). However, Grob (1949), working at lower enzyme concentration (10^{-9} M as compared with $1{\cdot}4 \times 10^{-7}$ M) and correspondingly higher degrees of dissociation, found $k = 6 \times 10^{-10}$ M, in reasonable agreement with our value. He also showed that trypsin inhibitors from ovomucoid and lima bean formed trypsin complexes which dissociated to similar extents, while the trypsin compound with soya inhibitor showed no dissociation.

It has not been possible to decide whether inhibition is competitive or non-competitive. The dissociation constant of the compound is very low compared to the known Michaelis constants for trypsin with a protein substrate (about 10^{-2} M, Fraenkel-Conrat *et al.* 1949) and if inhibition were competitive, it can be calculated that increase in haemoglobin concentration from 1 to 3% would only increase trypsin activity by 0·015 unit near the equivalence point, which would be scarcely detectable under the present experimental conditions. Also the conditions of test (incubation of enzyme with inhibitor before addition of substrate and short reaction time) are those under which even a true competitive inhibition would appear to be non-competitive (Wilson, 1951). It was decided to leave further work on this point until later, when ester substrates with low Michaelis constants (10^{-5} M, Schwert, Neurath, Kaufman & Snoke, 1948) will be investigated under different experimental conditions. (Preliminary work (Green, unpublished) has shown that, using ester substrates, competitive inhibition occurred with both pancreatic and soya-bean inhibitors.) A further disadvantage of the haemoglobin digestion method of measurement of trypsin activity, is that a strict kinetic interpretation cannot be applied to the results, owing to uncertainty over the value of S, and hence of K_m. The method is however useful in that the presence of urea slows the rate of reaction of enzyme and inhibitor so that the reaction velocity can be measured.

The marked effect of pH on the reaction between inhibitor and trypsin suggests that certain ionizing groups must be charged for the reaction to occur. Combination commences in the acid range between pH 3 and 4; the only ionizing groups in the inhibitor with pK in this range are the free carboxyl groups of aspartic and glutamic acids. In the alkaline pH range, ϵ-amino groups (pK 9·5–10·5) or guanidine groups (pK 11·5–12·5) might be playing a part in the reaction. In view of the fact that the inhibitor-trypsin compound is almost undissociated at pH 10·5 and only partially dissociated at pH 11·8, it appears that the dissociation of guanidine, rather than of ϵ-amino groups, is influencing the reaction. Alternatively, this pH effect might be due to the fact that in solution active trypsin is in equilibrium with a reversibly denatured form of trypsin; the extent of denaturation is dependent on pH and is a minimum at pH 5 (Kunitz & Northrop, 1934; Anson & Mirsky, 1934). If the inhibitor failed to combine with this reversibly denatured trypsin, the apparent degree of inhibition of trypsin would be dependent on pH. However, the extent of reversible denaturation at acid reactions is not sufficient to account for the observed retardation in rate of

formation of inhibitor-trypsin compound. In the alkaline region such an explanation in terms of denaturation would be more plausible.

The effect of pH on the combination of trypsin with other inhibitors has only been investigated for soya-bean inhibitor, here the compound dissociated below pH 2·9 but not between pH 3·6 and 10·4 (Sheppard, Maclaren & Johnson, 1950). It seems probable that most of the inhibitor-trypsin reactions are combinations between oppositely charged protein ions. Free amino and carboxyl groups have been shown to be essential for combination between trypsin and either ovomucoid or lima-bean inhibitors; when either of these inhibitors, or soya-bean inhibitor, combined with trypsin, there was a decrease in titratable amino nitrogen (Fraenkel-Conrat *et al.* 1949, 1952; Kunitz, 1947). With the exception of pancreatic inhibitor, all other inhibitors have been shown to react instantaneously with trypsin; pancreatic inhibitor takes a measurable time to react with trypsin in acid solution; it is also the only inhibitor known to have an isoelectric point in the alkaline region (Green & Work, 1953). Therefore, below pH 10, both trypsin (isoelectric point 10·8, Bier & Nord, 1951) and inhibitor will be positively charged, and so the combination between oppositely charged carboxyl and guanidine groups would be opposed by the net positive charge on the two molecules, and the reaction might be a relatively slow one. The reaction velocities have not yet been measured above pH 7, so that final proof of this explanation cannot be yet advanced.

The molar combining ratios with trypsin of the various types of inhibitors have not yet been fully worked out because of the present uncertainty as to the molecular weight of trypsin. Using ester substrates, Green (unpublished) has shown that 1 g. of a certain preparation of pancreatic inhibitor (activity 160) and 1 g. of soya-bean inhibitor were equivalent to 3·3 and 1·2 g. respectively of trypsin. These figures are in the right ratio for inhibitors of molecular weights 9000 and 24 000 respectively (Green & Work, 1953), and the pancreatic inhibitor figure is the same as the value found by Fraenkel-Conrat *et al.* (1952) for the lima-bean inhibitor which also has a molecular weight of about 9000.

SUMMARY

1. The combination of pancreatic trypsin inhibitor with trypsin was investigated. The dissociation constant of the inhibitor-trypsin compound was determined, using haemoglobin substrate.

2. The degree of inhibition of trypsin was unaffected by fourfold variation of substrate concentration.

3. A method was developed for determining the extent of reaction in an inhibitor-trypsin mixture. The velocity constants of the reaction in the presence and absence of substrate were determined.

4. The inhibitor-trypsin combination was considerably retarded by the presence of urea.

5. The presence of denatured trypsin was without effect on the reaction.

6. The effect of pH on reaction rates was investigated. At pH's 4 and 12·4 the degree of combination after 1 min. was negligible, at pH 3 no measurable reaction was found to occur even after several hours, while at pH's 10·5 and 7 combination was almost complete in 1 min.

7. An explanation of the slow rate of reaction and its pH dependence is advanced in terms of ionic combination, considering that both proteins have alkaline isoelectric points.

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